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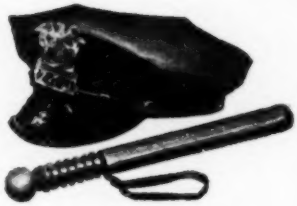

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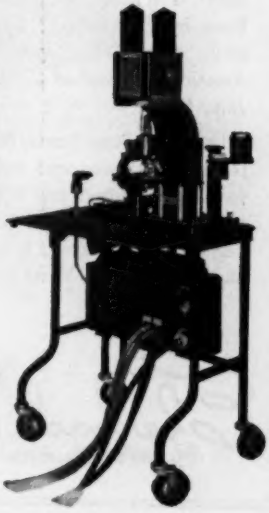

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INFARCTION OF THE BRAIN WITHOUT THROMBOSIS

An Analysis of One Hundred Cases with Autopsy

SAMUEL P. HICKS, M.D.
AND
SHIELDS WARREN, M.D.
BOSTON

APOPLEXY is the third leading cause of death in this country, but there is much to be learned about it. Among the most common misconceptions are those still widely taught, that infarcts of the brain are usually caused by thrombosis and that most cerebral hemorrhages result from rupture of sclerotic arteries. Indeed, the distinction between hemorrhage and infarction, though clinically and descriptively useful, is usually arbitrarily based on the gross pathologic picture rather than on the underlying vascular process. Despite common observation and considerable published literature to the contrary, general practitioners and specialists alike are often inclined to view the patient with prodromal symptoms of stroke or actual apoplexy as a relatively hopeless prospect, and one in whom only watchful expectancy will determine the prognosis. The statement might be ventured that because of this pessimistic viewpoint about the irreversibility of the pathologic processes that determine cerebral vascular accident, little has been done about preventive therapy.

That rupture of arteries and thrombosis are not the cause of many strokes is not a new concept. The cause of cerebral hemorrhage puzzled students as early as Morgagni [1] (1789) and Charcot [2] (1890), who surmised on indirect evidence that aneurysms might be the causative lesions. To Rokitansky [3] in 1856 hemorrhage and infarction were caused by several processes. Rosenblath [4] in 1918 pointed out that cerebral hemorrhage was a result of bleeding from many small necrotic vessels. Westphal and Baer [5] in 1926 took a similar view and concluded that vasospasm brought about necrosis of parenchyma and small vessels, a conclusion increasingly well supported by other evidence accumulated in recent years. As a result of a

From the Departments of Pathology of the New England Deaconess Hospital, the Peter Bent Brigham Hospital, and the Harvard Medical School.

1. Morgagni, J. B.: The Seats and Causes of Diseases Investigated by Anatomy, Translated from the Latin by Benjamin Alexander, London, Millar & Cadell, 1769.

2. Charcot, J. M.: Hémorrhagie et ramollisement du cerveau, in Oeuvres completes de J. M. Charcot, 1890, Vol. 9, p. 10.

3. Rokitansky, C.: Abnormitäten des Nervensystem: Hämorrhagie, in Lehrbuch der pathologischen Anatomie, Vienna, Wilhelm Braumuller, 1856, Vol. 2, p. 443.

4. Rosenblath: Über die Entstehung der Hirnblutung bei dem Schlaganfall, Deutsche Ztschr. Nervenh. **61**:10, 1918.

5. Westphal, K., and Baer, R.: Über die Entstehung des Schlaganfalles: L. Pathologisch-anatomische Untersuchungen zur Frage der Entstehung des Schlaganfalles, Deutsches Arch. klin. Med. **151**:1, 1926.

critical review of literature Stern in 1938 stated "it is quite evident that necrotic lesions of the brain may occur without any associated visible obstruction (thrombosis, embolism, obliteration) of the arteries." [6] In this country, among others, Scheinker [7] de-emphasized the ruptured artery as a cause of hemorrhagic infarction and showed that the condition could result from confluence of blood from many small vessels, especially the deep cerebral venules. He attributed this to a form of vasoparalysis.

In a recent study of the relation of cardiovascular disease and apoplexy Hicks and Black [8] reviewed 155 cases of apoplexy with autopsy, 118 of which were of the hemorrhagic type. They showed that, aneurysms excepted, hemorrhage usually arose from many ischemically damaged or necrotic small vessels in hypertensive subjects and was frequently unrelated to arteriosclerosis. In a series of 23 patients whose fatal lesions were predominantly infarction, they found 11 without mechanical vascular occlusion. In only two instances was it possible to implicate a drop in systemic blood pressure as the cause of the ischemic brain lesions. From these and other observations they concluded that arterial spasm accounted for most of these cases of apoplexy and that cerebral vasospasm seemed frequently to be a component of the hypertensive state.

That arterial spasm occurs in the human brain is attested by direct and indirect evidence. By spasm is meant muscular constriction of arteries and arterioles, though not necessarily to the point of complete occlusion. Arterial spasm has been directly observed in some patients during intracranial operations.[9] Real constrictive blanching of retinal arteries,[10] which are part of the arterial system of the brain, likewise is seen. Indirect but suggestive evidence is found in the cat brain, in which mechanical stimulation may cause visible focal arterial constriction.[11] Villaret and Cachera [12] introduced tiny emboli experimentally into the arterial circulation of the dog's brain. They observed that occlusion of tiny arteries caused not only local vasoconstriction but arterial spasm in other arterial branches. This remote reflex spasm, which lasted from hours to weeks, led to infarction and hemorrhage in the affected regions.

---

6. Stern, K.: The Pathology of Apoplexy: A Critical Review, J. Neurol. & Psychiat. **1**:26, 1938.

7. Scheinker, I. M.: Alterations of the Cerebral Veins in Hypertensive Brain Disease and Their Relation to Cerebral Hemorrhage, Arch. Neurol. & Psychiat. **54**:395 (Nov.-Dec.) 1945.

8. Hicks, S. P., and Black, B. K.: The Relation of Cardiovascular Disease to Apoplexy: A Review of 155 Cases with Autopsy, Am. Heart J. **38**:528, 1949.

9. Penfield, W.: Circulation of the Epileptic Brain, in Circulation of the Brain and Spinal Cord: A Symposium on Blood Supply, A. Res. Nerv. & Ment. Dis., Proc. **18**:605, 1938.

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12. Villaret, M., and Cachera, R.: Les embolies cérébrales: Études de pathologie expérimentale sur les embolies solide et gazeuse du cerveau, Paris, Masson & Cie, 1939.

The reversible nature of hypertensive encephalopathic attacks, their relief by papaverine,[13] and the sometimes beneficial effect of stellate ganglion block [14] early in the course of a stroke add further evidence to the vasospastic concept. In a number of clinics, transient block in blood flow through branches of the circle of Willis has been demonstrated in hypertensive subjects by arteriogram. Poppen [15] and his associates have demonstrated by carotid and vertebral arteriogram failure of certain principal branches of the circle of Willis to fill with radiopaque material in selected apoplectic patients preparatory to stellate ganglionectomy. In two of their cases the apoplexy was fatal. At autopsy the unfilled artery in the arteriogram in one case was normal, and the infarct was in the distribution of another morphologically undiseased artery. In the other case, arteriosclerosis was present, but the vessel was not occluded and the infarct corresponded to this vessel. In neither case was the lack of filling due to an anomaly. In a clinic where this procedure is frequently performed, failure to visualize the vessel in the absence of anomaly or mechanical obstruction suggests vasoconstriction (spasm) in the subdivisions of that branch at the time of the arteriogram, sufficient to reduce blood flow in the vessel.

That the cerebral arteries in hypertensive vascular disease are in a general state of vasoconstriction greater than normal has been shown in a number of studies by Kety and associates [16] by determinations of cerebral blood flow. However, these workers observed no net increase in blood flow after stellate ganglion block, spinal sympathetic block, or sympathectomy, although diminished vascular resistance was observed that partially compensated for reduction in systemic arterial blood pressure. It is important to note in these studies by Kety and associates that patients with imminent or incipient stroke were not studied and that it remains to be determined how much the diminished blood flow in one part of the arterial tree accompanying a stroke—for a stroke is usually a consequence of ischemia [8]—would be reflected in blood flow measurements.

The first purpose of the present study is to review certain pathologic and clinical findings in a series of patients dead with infarctive apoplexy, which was sufficiently large and studied carefully enough that some valid conclusions about the pathogenesis of the condition can be drawn; and to compare the findings where pertinent to those in a previously reported large series of patients dead of hemorrhagic apoplexy.[8] The second purpose is to emphasize by this means that cerebral infarction is often a result of ischemia due not to thrombosis but to nonmechanical vascular obstruction which may in future be amenable to measures of preventive medicine.

---

13. Russek, H. I., and Zohman, B. L.: Papaverine in Cerebral Angiospasm (Vascular Encephalopathy), J. A. M. A. **136**:930 (April 13) 1948.

14. (*a*) Leriche, R., and Fontaine, R.: De l'infiltration stellaire dans les embolies cérébrales, dans les spasmes vasculaires postopératoires de l'encéphale et chez les hémiplégiques, Rev. chir., Paris **74**:751, 1936. (*b*) Gilbert, N. C., and de Takats, G.: Emergency Treatment of Apoplexy, J. A. M. A. **136**:659 (March 6) 1948. (*c*) Mackey, W. A., and Scott, L. D. W.: Treatment of Apoplexy by Infiltration of Stellate Ganglion with Novocain, Brit. M. J. **2**:1, 1938.

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16. Kety, S. S., and others: Blood Flow, Vascular Resistance, and Oxygen Consumption of the Brain in Essential Hypertension, J. Clin. Invest. **27**:511, 1948; Effects of Acute Reduction in Blood Pressure by Means of Differential Spinal Sympathetic Block on Cerebral Circulation of Hypertensive Patients, ibid. **29**:402, 1950. Harmel, M. H., and others: Effect of Bilateral Stellate Ganglion Block on the Cerebral Circulation in Normotensive and Hypertensive Patients, ibid. **28**:415, 1949.

MATERIAL FOR STUDY

The 100 cases for this study were obtained for the most part from the autopsies and records of the departments of pathology of the New England Deaconess Hospital and the Peter Bent Brigham Hospital, in Boston, in the years 1930 to 1951. In addition some cases were studied at the departments of pathology of the Naval Medical School, Bethesda, Md., and the Georgetown University Medical Center, Washington, D. C., in the years 1945 to 1948.[8] Cases were selected in which the stroke was the principal cause of death or was one of the prime causes of death—for example, in five instances coronary thrombosis possibly sufficient in itself to cause death was also present. Terminal bronchopneumonia was often a contributory factor in death. Only those cases were selected in which the infarct or infarcts were so large that the blood vessels supplying the region could be and were adequately studied in the gross, and in which the lesion was clearly the result of reduction or occlusion of blood flow in one or more major cerebral arteries. On these bases it was necessary to exclude only a few cases of large or massive infarction; in several instances the examination of the vessels was not considered to be adequate for this study. Two cases of subacute bacterial endocarditis were not included because abscess formation obscured the vascular relations. No cases of syphilis were found, but several instances of mitral stenosis were. Three cases in which there was venous thrombosis and two cases in which there was ligation of the internal carotid artery were excluded. No other examples of occlusion of the internal carotid artery were found, although in the arteriosclerotic group this vessel was sometimes involved by sclerosis along with other intracranial arteries. Two cases were excluded because only the head was studied. With rare exceptions, infarction was two to several days old. In two instances the patients lived several weeks; in them thrombosis was the cause of the stroke. In three cases the infarction could almost certainly be attributed to therapeutically induced hypotension—actual prolonged shock of severe degree.

Altogether, the sampling comprises most of the cases of extensive and fatal cerebral infarction in the autopsy series noted above. Like any series, this one is weighted in several ways. Small and old infarcts are not included, though there is presently no evidence that small lesions have different pathogeneses from large ones. The nature of the hospital is a determinant; the New England Deaconess Hospital weights the series with 15 of a total of 16 patients with diabetes where that disease is commonly seen. At the Peter Bent Brigham Hospital mitral stenosis is fairly frequently seen, and all of the probable embolic cases came from that institution. All but three patients were of the white race. Consideration of 100 cases, however, should minimize some of these factors and offer a fair picture of what are the commoner mechanisms involved in infarctive apoplexy.

All autopsies were complete. The vessels of the brain were studied by external examination and dissection in the fresh state, and then further examination was carried out either after the whole brain had been fixed in 4% formaldehyde solution (10% formalin®) or immediately, without fixation. In a few instances, when there was a question of postmortem clot versus antemortem thrombus, serial sections were made of the doubtful lesion. Note was made of whether the thrombus accounted for the entire extent of infarction. In seven cases the thrombus could not have accounted entirely for the infarction even if one allowed for maximal overlap and variations in the major cerebral arteries; or there were two or more infarcts, with only one caused by a thrombus. The presence, the absence, and the degree of arteriosclerosis were noted in grossly visible arteries and in those of microscopic size. Routine microscopic studies of the infarcted regions and the unaffected regions were carried out in all cases. In addition to findings in the brain the following observations were recorded: age, sex, race, history of vascular hypertension, heart weight, coronary arterial disease, cardiac valvular disease, presence of auricular or mural cardiac thrombi, evidence of embolism, and the presence of congestive heart failure, shock, or other circulatory disturbances in relation to the onset of the apoplectic episode.

Comment on the criteria for selecting these cases as illustrating infarction as opposed to cerebral hemorrhage is essential to the thesis of this presentation, for it is common practice to consider these conditions as separate diseases. Actually in a large series of cases of apoplexy, exclusive of bleeding aneurysms and that entity virtually never demonstrated, the "ruptured sclerotic artery," a spectrum is formed with massive hemorrhage on the one hand and nearly bloodless necrosis on the other, with all shades between.[17] In all cases of hemorrhage of this

17. Globus, J. H.: Massive Cerebral Hemorrhage, in Circulation of the Brain and Spinal Cord: A Symposium on Blood Supply, A. Res. Nerv. & Ment. Dis. **18**:438, 1938.

sort in these laboratories,[18] the blood present derived from coalescence of hemorrhages of many partly damaged or frankly necrotic small vessels and was accompanied by varying degrees of infarction of the parenchyma, sometimes early, sometimes well advanced.

It is important to establish here that hemorrhagic apoplexy and infarction are variations of a single underlying ischemic process. This can be brought out by three examples (not included in the 100 cases) that illustrate the pathogenesis of the hemorrhagic process that has characterized a very large number of cases in these laboratories. In the first instance a woman aged 60 was being operated on for the removal of a salivary gland tumor and it was necessary to occlude the internal carotid artery on the left for a period stated to be five minutes. This resulted in severe left cerebral infarction, with death in a few days. There was necrosis in the upper outer striatal region, the insula, and the lateral portion of the cerebral hemisphere in the distribution of the middle cerebral artery and the deep anterior striatum in the distribution of branches of the anterior cerebral artery. The head of the caudate nucleus was markedly hemorrhagic, and several hemorrhagic zones developed in the affected cortical gray and subcortical white matter. The ventricular fluid was blood-tinged. The lesion presented itself principally as an infarct. The cerebral and internal carotid arteries were completely free of arteriosclerosis. In a second instance, in a 24-year-old man thrombosis developed in a small, unruptured aneurysm of the left anterior cerebral artery, just at its origin from the internal carotid artery, which seriously compromised blood flow in that anterior artery and partially that in the middle cerebral artery. The result was infarction generally similar to that of the first case but more hemorrhagic and accompanied by extensive intraventricular hemorrhage. The third case [19] is one of a 70-year-old hypertensive Negro who had extensive multiple confluent hemorrhages and infarctive lesions throughout the whole right hemisphere, massive intraventricular hemorrhage, minimal hemorrhagic infarcts in the left hemisphere, and a new thrombus in the right middle cerebral artery. There was moderate cerebral arteriosclerosis.

Clearly, the distinction among these cases lies mostly in the gross descriptive aspects of the lesions, whereas there is a fundamental vascular process—ischemia—common to all three. The reasons why at one time a lesion is principally infarctive and at another is hemorrhagic is only partly understood.[18a] Overlapping blood supply to the affected region, degree and duration of the ischemia, arteriosclerosis of collateral vessels, increased capillary permeability, changes in extravascular and intravascular pressure and in blood flow undoubtedly play parts. Age is also a general factor. In the present series of cases of infarction only 21 of 100 patients were under 50 years. In the previous series of 118 cases of hemorrhage [8] 41% were under the age of 50 and they usually had little or no arteriosclerosis.

Classification of cerebral vascular accidents then becomes arbitrary; of these three cases, the second and third would be classed as cerebral hemorrhage, the first as infarction. The selection of predominantly infarctive lesions for this study was made therefore because many aspects of the vascular pathologic process lend themselves to easier demonstration than is the case in hemorrhagic types.

ANALYSIS OF RESULTS

Since it is a major aim of this report to invite attention to the frequency with which brain infarction occurs *without* thrombosis or mechanical occlusion, findings and discussions are directed to factors that bear on this phenomenon. A number of pertinent data are summarized in Tables 1 and 2. Some data are represented by only a few cases, so that their inclusion is to be construed as serving the purpose of completeness rather than numerical significance.

Ischemic lesions in this series could be caused by several processes: primary thrombosis of an artery; embolic obstruction of an artery; arteriosclerotic obstruction of an artery; systemic circulatory failure, such as heart failure, shock, or rela-

18. (*a*) Hicks, S. P., and Warren, S.: Introduction to Neuropathology, New York, McGraw-Hill Book Company, Inc., 1950, Chap. 2. (*b*) Hicks and Black.[8]

19. This case is illustrated in Hicks and Warren's book,[18a] p. 70.

tive hypotension in the hypertensive state, and functional changes in the arterial tree, such as spasm, that lead to partial or nearly complete occlusion and ischemia. Thrombosis of cerebral vessels accounted completely for the infarct in 33 cases and accounted partially for that in an additional seven cases. Sixty patients, then, had no thrombosis, and a total of 67 cases—two-thirds—require some other explanation than the one usually given, namely thrombosis.

How many of the cerebral-thrombotic patients derived their vascular occlusion from thrombotic emboli is not known. Cardiovascular disease of some kind was

TABLE 1.—*Cardiovascular Findings in One Hundred Cases of Brain Infarction*

Infarction with no cerebral thrombosis		60
With heart or other circulatory failure	21	
With vascular hypertension	43	
With severe or moderate cerebral arteriosclerosis	34	
With minimal or no cerebral arteriosclerosis	22	
With thrombi in heart	4	
With coronary thrombosis	3	
With diabetes	10	
Infarction wholly due to cerebral thrombosis		33
With heart or other circulatory failure	18	
With vascular hypertension	21	
With severe or moderate cerebral arteriosclerosis	9	
With no cerebral arteriosclerosis	1*	
With thrombi in heart (embolism?)	10	
With coronary thrombosis	2	
With diabetes	4	
Infarction partially explained by thrombosis		7
With heart or other circulatory failure	5	
With vascular hypertension	6	
With severe or moderate cerebral arteriosclerosis	4	
With thrombi in heart (embolism)	1	
With minimal or no cerebral arteriosclerosis	3	
With diabetes	2	

* Mitral stenosis with embolism, 1.

TABLE 2.—*Cardiovascular Findings in the Thirty-Four Cases of Nonthrombotic Brain Infarction with Severe to Moderate Cerebral Arteriosclerosis*

Infarction with severe to moderate cerebral arteriosclerosis, but no thrombosis		34
With heart or other circulatory failure	13	
With vascular hypertension	17	
With thrombi in heart	3	
With combined vascular hypertension and heart failure	13	

common in this series of cases as it was in the previously reported series of hemorrhage [8] and in the very large series reported recently by Wilson, Rupp, Riggs, and Wilson.[19a] Some degree of coronary arteriosclerosis was present in 73. There was heart failure in 44; in five of these it was associated with acute myocardial infarction and in six with mitral stenosis. In only 15 cases were there thrombi in the heart at autopsy, and seven of these had valvular disease. In five of these seven cases embolism can reasonably be stated to have caused the cerebral vascular

19a. Wilson, G.; Rupp, C.; Riggs, H. E., and Wilson, W. W.: Factors Influencing the Development of Cerebral Vascular Accidents, J. A. M. A. **145**:1227, 1951.

occlusion. This suggests that large emboli that occlude major cerebral arteries account for only relatively few cases of cerebral vascular occlusion and then especially in the presence of valvular disease. Whether minute emboli, impossible of identification, may induce widespread reflex arterial spasm as they do in the experimental dog,[12] must remain presently speculative.

Arteriosclerosis may play two very important roles in the genesis of cerebral infarction. The most obvious is that a sclerotic artery may tend to become thrombosed, and this was the explanation in the cases of thrombosis. The other role is that of partial obstruction with the blood flow reduced to the extent that a thrombus in a small branch of such a sclerotic tree might precipitate infarction; or under the same circumstances systemic circulatory failure might induce infarction in the distribution of the most seriously obstructed part of the cerebral circulation. This study showed only one reasonable example of this sort, but a dozen others might have occurred in this way (Table 2).

The possible role of systemic circulatory failure in producing apoplexy may now be examined. At the outset it may be stated that systemic arterial hypotension, to produce cerebral infarction, would have to act on a brain whose blood supply is already compromised (for example, by obstructing arteriosclerosis), or it would have to be so profound and prolonged as to produce severe general anoxia. It is a matter of common observation both at the bedside and in the operating room that most people who have coronary occlusion, congestive heart failure, or circulatory shock do not have apoplexy if their cerebral arteries are normal or not seriously embarrassed by arteriosclerosis. The brain is able to withstand a considerable degree of anoxemia and of reduced cerebral blood flow up to a critical point.[18a] In this series there was not consistent correlation between heart failure and the formation of infarcts. As seen in Tables 1 and 2, systemic circulatory failure could not account directly for most of the apoplexies, simply because it was present in only about one-third of the nonthrombotic cases. The hypertensive state was more frequently present, but interestingly enough it was present in only 50% of the cases of nonthrombotic infarction with marked cerebral arteriosclerosis. Mere association of two disease conditions does not imply a cause and effect relation, but both systemic circulatory failure and hypertension have been rationally related to apoplexy. Circulatory failure has occasionally been a precipitant of apoplexy [8] but is not often proved to be related. Hypertension, however, has shown a much higher associative correlation, and from the pathogenetic viewpoint has been closely related by the observations on hypertensive cerebral vasoconstriction [16] and encephalopathy.[20] Circulatory failure and vascular hypertension accompanied only 17 of 34 cases in the subgroup of nonthrombotic but sclerotic cases of infarction, so that an intrinsic cerebral vascular disease has to be implicated in at least 17. Arteriosclerosis is a slowly developing process and cannot account for this intrinsic process, so that an explanation must lie in some rapid local changes in blood flow leading to ischemia—constriction of the finer arterial branches is the only answer.

Of collateral interest is the possibility that systemic circulatory failure might, by slowing blood flow, contribute to the formation of cerebral vascular thrombi. Of the total of 40 patients with thrombosis, 23 had heart failure, and 11 of these 23 had

20. Russek and Zohman.[13] Leriche and Fontaine.[14a] Gilbert and de Takats.[14b] Mackey and Scott.[14c]

thrombi in the heart or had mitral stenosis. From this it is concluded that heart failure is not essential to the formation of primary cerebral thrombi, for 17 patients had no failure.

The relation of the hypertensive state to the genesis of apoplexy appears to be a statistically significant one and from the pathologic viewpoint equally significant. In this series 70 patients had high blood pressure, that is, a history of sustained hypertensive levels in excess of 90 mm. Hg diastolic and 140 mm. systolic pressure. Most were considerably over these levels and had had the disease for one to a number of years. In two additional patients systolic pressure was abnormally high but the diastolic pressure was below 90; these were not counted as having hypertensive vascular disease. In the previous series[8] of 118 cases of cerebral hemorrhage (aneurysms excluded) the incidence of arterial hypertension was nearly 90%; the explanation for most of the lesions lay in a functional change in the small vessels of the brain, since cerebral arteriosclerosis and severe systemic circulatory failure were usually excluded as causes.

In the present series the same process seemed to be operative in most of the nonthrombotic cases. The lesions were ischemic, and there was no cerebral vascular occlusion; systemic circulatory failure was excluded as a cause in most cases, so that the remaining possibility was intrinsic focal reduction of blood flow. Since there were also necrosis, evidence of stasis, and sometimes diapedesis in both small arteries and venules as well as capillaries, arterial constriction must have been the cause.

It is probable that not a great degree of constriction (spasm) is necessary to produce the ischemia, for interstitial edema is an outstanding gross and microscopic characteristic of apoplectic lesions. Edema is a serious condition in brain tissue, for the integrity of cerebral blood flow depends much on a sharp differential between a relatively high intravascular pressure and a brain tissue pressure which in the erect position in man is below atmospheric pressure.[21] A little leakage of fluid into the parenchyma rapidly lessens this gradient to a point where tissue pressure impairs blood flow, anoxia becomes worse, severe ischemia ensues, edema increases, and a progressively vicious cycle develops. Thus in the brain comparatively slight vascular ischemia may often readily be converted into a serious irreversible lesion. In this connection it is pertinent to note that sodium chloride retention is an integral part of congestive heart failure and, as recently demonstrated quantitatively by Thorn,[22] often a part of the hypertensive state. This may be one of the important contributory factors in the genesis of apoplexy, for in patients with sodium retention edema would be expected to develop more readily than in others.

The possibility that disease of the internal carotid artery might contribute to the development of apoplexy cannot be excluded in all of these cases. Although the bony part of the internal carotid arteries is not usually dissected out completely in routine autopsies, a sufficient number were studied in this series to exclude carotid obstruction as more than a rare cause of apoplexy. Severe carotid sclerosis might have been a contributing factor when present in some cases with severe circulatory failure, but it would be subject to the same limitations as noted elsewhere in this report.

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21. Fremont-Smith, F., and Merritt, H. H.: The Cerebrospinal Fluid, Philadelphia, W. B. Saunders Company, 1938. Hicks and Warren.[18a]

22. Thorn, G. W.: The Effects of Bilateral Adrenalectomy in Essential Hypertension, to be published.

COMMENT

The crux of the problem of determining the principal mechanism responsible for most cerebral vascular accidents lies in considering the nature of infarctive and hemorrhagic lesions that are not caused by mechanical vascular obstruction. Ischemia is the cause of virtually all of these accidents (excluding aneurysm) whether they are hemorrhagic or predominantly nonhemorrhagic, and whether thrombosis is present or not. The problem then comes down to whether the focal lack of blood flow—ischemia—is due to a local change in vascular caliber, such as spasm or vasoparalysis,[7] or whether it is a result of systemic circulatory failure. In the 33 cases of thrombosis, the mechanism is obviously local. Out of the 67 cases in which lesions were not caused by thrombosis in this series and almost all of the previous series of 118 hemorrhagic cases,[8] systemic circulatory failure could be implicated as a prime cause in only 3. In an indeterminate number, possibly a total of 13, systemic failure superimposed on cerebral arteriosclerosis might have been responsible for the lesions. Conversely, then, an intrinsic vascular change must be responsible in most instances. That it is primarily arterial in nature is evidenced by necrosis of the arteriolar side of the vascular tree as well as of the capillary and venular side. Were the stasis primarily in the venous side, the small arteries would be less often affected.

The mechanism by which normal degrees of vasoconstriction and dilatation occur in the brain of man is not completely known. There is evidence that the smooth muscle of the cerebral arteries and arterioles tends to constrict spontaneously unless acted on by carbon dioxide, the direct action of which causes vasodilation.[23] It has been abundantly demonstrated that the superficial and the penetrating arteries of the human brain are well supplied by nonmyelinated nerves derived from at least two sources, the vertebral arterial and internal carotid arterial plexuses (McNaughton).[24] That they stem from the stellate ganglion is partly established, but it is not known whether other plexuses contribute fibers, or whether any fibers in the deep arteries are sensory. Volpitto and Risteen[25] have observed, through burr holes, ipsilateral dilation of the superficial cerebral arteries in man following stellate ganglion block, which suggests a direct nervous pathway of control from that center. These observations demonstrate that there are mechanisms that can bring about a degree of vasoconstriction.

Altogether it would seem that apoplexy is a disease complex with a number of causes and contributory factors, but in most instances the finger points to an intrinsic local vascular disturbance which on pathologic evidence appears to be arterial constriction. In most instances, all forms of ischemic apoplexy being considered, this is associated with the hypertensive state; in a smaller number circulatory failure or severe arteriosclerosis of the brain is present. Whether these various

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23. Cobb, S.: The Cerebral Circulation, in Circulation of the Brain and Spinal Cord: A Symposium on Blood Supply, A. Res. Nerv. & Ment. Dis. **18**:719, 1938. Weiss, S., and Lennox, W. G.: The Cerebral Circulation: XVII. Cerebral Blood Flow and Vasomotor Response of Minute Vessels of Human Brain to Histamine, Arch. Neurol. & Psychiat. **26**:737 (Oct.) 1931.

24. McNaughton, F. L.: Innervation of Intracranial Blood Vessels and Dural Sinuses, in Circulation of the Brain and Spinal Cord: A Symposium on Blood Supply, A. Res. Nerv. & Ment. Dis. **18**:178, 1938.

25. Volpitto, P. P., and Risteen, W. A.: Use of Stellate Ganglion Block in Cerebral Vascular Occlusions, Anesthesiology **4**:403, 1943.

conditions may subtly alter cerebral blood flow and, by the mechanism of partial anoxia, precipitate vasospasm, as happens elsewhere in the body (Trueta),[26] is not known.

SUMMARY AND CONCLUSIONS

A series of 100 cases of fatal cerebral infarction with autopsy is analyzed with emphasis on the pathologic physiology involved in the nonthrombotic cases. It is compared with a similar series of cases of cerebral hemorrhage previously reported.[18] In 60 of the 100 cases of infarction there was no mechanical occlusion of cerebral vessels by thrombosis, embolism, or arteriosclerosis and thus some other explanation is required for the infarction. The factors possibly responsible, such as cerebral arterial spasm, systemic circulatory failure, severe arteriosclerosis, and vascular hypertension are examined. It is emphasized that virtually all forms of apoplexy (exclusive of aneurysm), whether chiefly hemorrhagic or predominantly infarctive, have a common basis in local cerebral ischemia. In most nonthrombotic cases this is explained by local reduction of blood flow which, on pathologic and clinical bases, is best attributed to vasoconstriction (spasm). In only a few patients could systemic circulatory failure (shock, heart failure) coupled with severe cerebral arteriosclerosis be implicated as a primary cause of the stroke. The complicating role of cerebral edema is discussed and the suggestion made that sodium retention in heart failure and the hypertensive state may often have been an aggravating factor.

It is concluded that apoplexy is an ischemic disease of the brain most often directly caused by cerebral vasospasm, less often by thrombosis, and least often by systemic circulatory failure, embolism, and arteriosclerosis.

26. Trueta, J., and others: Studies of the Renal Circulation, Springfield, Ill., Charles C Thomas, Publisher, 1947.

STUDIES ON THE "ALKALINE" PHOSPHATASE ASSOCIATED WITH REGENERATING CONNECTIVE TISSUE FIBERS

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IN AN EARLIER investigation Gold and Gould [1] demonstrated that the alkaline phosphatase localized on regenerating connective tissue in healing skin wounds by Fell and Danielli [2] is intimately bound to the fibers themselves. They also demonstrated that freshly precipitated collagen fibers firmly bind phosphatase. Bourne [3] demonstrated that healing bone wounds of the guinea pig exhibit strong alkaline-phosphatase activity during the deposition of the fibrous bone matrix. Johnson, Butcher, and Bevelander,[4] Bradfield,[5] and Jeener [6] showed that the synthesis of certain fibrous protein appears to be accompanied with marked phosphatase activity. However, Robertson, Dunihue, and Novikoff,[7] studying fibrous capsule formation about guinea-pig kidney, found that collagen fiber formation was not accompanied with high phosphatase activity. Further evidence is desirable, therefore, to demonstrate whether or not there is a relationship between phosphatase activity and fibrogenesis.

In scorbutic guinea pigs, in which regeneration of connective tissue is very poor, Danielli, Fell, and Kodicek [8] and Bourne [3b] showed that there is no marked increase of phosphatase activity in wounds such as appears in wounds of normal guinea pigs. These observations, together with the fact that the serum phosphatase levels of scorbutic guinea pigs are very low, as shown by Scoz, Cataneo, and Gabrielli,[9] Todhunter and Brewer,[10] and Shwachman and Gould,[11] suggested that the failure of scorbutic animals to produce connective tissue in skin wounds might be due to absence of blood phosphatase. In this respect it is interesting that the rat, in which tissue regenerates rapidly, has a high blood phosphatase level. On

From the Department of Biology, Massachusetts Institute of Technology.

1. Gold, N., and Gould, B. S.: Arch. Biochem. & Biophysics **33**:155, 1951.
2. Fell, H. B., and Danielli, J. F.: Brit. J. Exper. Path. **24**:196, 1943.
3. Bourne, G.: (*a*) Quart. J. Exper. Physiol. **32**:1, 1943; (*b*) J. Physiol. **102**:319, 1943.
4. Johnson, P. L.; Butcher, E. O., and Bevelander, G.: Anat. Rec. **93**:355, 1945.
5. Bradfield, J. R. G.: Nature **157**:876, 1946.
6. Jeener, R.: Nature **159**:578, 1947.
7. Robertson, W. V.; Dunihue, F. W., and Novikoff, A. B.: Brit. J. Exper. Path. **31**:545, 1950.
8. Danielli, J. F.; Fell, H. B., and Kodicek, E.: Brit. J. Exper. Path. **26**:367, 1945.
9. Scoz, G.; Cataneo, C., and Gabrielli, M. C.: Enzymologia **3**:29, 1937.
10. Todhunter, E. N., and Brewer, W.: Am. J. Physiol. **130**:310, 1940.
11. Shwachman, H., and Gould, B. S.: J. Nutrition **23**:271, 1942.

the other hand, the failure to find phosphatase in the wounds of scorbutic animals may be due to the absence of fibers, which may be needed to adsorb the enzyme.

If healing of the wound is dependent on the local phosphatase level, which in turn may be related to the blood phosphatase level, then the rate of wound healing might be expected to be modified by increasing or decreasing the serum phosphatase level. It should at the same time be possible to determine whether there is a direct relationship between serum phosphatase and wound phosphatase. Methods are available for altering the serum alkaline-phosphatase levels in rats. Weil and Russell,[12] using rats, have shown that fasting markedly lowers the phosphatase activity of the serum. Gould [13] observed that extensive fat feeding leads to highly elevated blood-serum alkaline-phosphatase levels. It is possible, therefore, to obtain rats with low, normal, and high serum-phosphatase values and to observe the rate of healing and the wound phosphatase intensities in such animals.

The possibility that fibrogenesis depends on phosphatase might be studied in yet another way. Alkaline phosphatase has been administered to wounds in an attempt to stimulate healing by Blum,[14] who concluded that an alkaline phosphatase preparation injected along with calcium glycerophosphate (in an alginate "anchoring" medium) hastened the healing of artificially produced bone gaps in the rabbit. He also observed the formation of ectopic bone in muscle into which alkaline phosphatase, calcium glycerophosphate, and "anchoring" medium had been introduced. It appeared, therefore, that in the presence of an alginate carrier a "foreign" alkaline phosphatase was sufficient to stimulate the regeneration of bone at a rate greater than normal. Robertson [15] showed that the subcutaneous injection of alginic acid, the carrier base used by Blum,[14] stimulates fibrogenesis in the normal guinea pig but not in the scorbutic guinea pig. These results suggest that phosphatase be used in an alginate anchoring medium in skin wounds of scorbutic animals as a means of determining the possible role of phosphatase in fibrogenesis.

Another approach to determining a role for phosphatase in fibrinogenesis is that of repeatedly administering powerful phosphatase inhibitors to skin wound areas to determine whether inhibition of the phosphatase activity of the area definitely retards or prevents fiber regeneration.

The results of this investigation indicate the effect of altered serum alkaline-phosphatase levels on the rate of healing of skin wounds and on the amount of alkaline phosphatase present in the wound areas, as well as the effects on wound healing and fiber formation of introducing relatively massive amounts of enzyme or potent phosphatase inhibitors into the areas of regeneration. They cast some doubt on the significance of the wound phosphatase in fibrogenesis.

EXPERIMENTAL PROCEDURES

Alteration of Serum Phosphatase Levels

Animals.—Albino rats were used. The animals were divided into three groups: (1) those whose wounds were allowed to heal for two days before excision; (2) those whose wounds were allowed to heal for seven days before excision of the regenerating tissue; and (3) those whose wounds were allowed to heal for nine days. Each group was subdivided into three

12. Weil, L., and Russell, M. A.: J. Biol. Chem. **136**:9, 1940.
13. Gould, B. S.: Arch. Biochem. **4**:175, 1944.
14. Blum, G.: Lancet **247**:75, 1944.
15. Robertson, W. V.: Personal communication to the authors, 1949.

dietary groups: (1) high fat, (2) normal, and (3) fasted. Water was present at all times. All animals except those being fasted were fed purina® checkers[16] ad libitum. The fat-fed animals were given in addition 3 ml. of cod liver oil by medicine dropper three times daily for five days prior to wounding and in the same dosage throughout the period of healing. The fasted animals were wounded two days after the start of the fasting period.

Production of Wounds.—The animals were anesthetized lightly with ether prior to wounding. The skin was plucked clean of hair in a small area on both sides just posterior to the ribs. A circle was cut on the skin about ¼ in. (6 mm.) in diameter with a cylindrical blade rotated by hand. The circle of skin was then excised down to the panniculus carnosus. The wounds were not dressed, but no infections were noted. The partially healed wounds surrounded by some normal tissue were excised for study. The tissues were dehydrated in alcohol and embedded in paraffin.

Serum-Phosphatase Determinations.—These were carried out essentially by the method of Shinowara, Jones, and Reinhart[17] as modified by Gould and Shwachman.[18] Blood (about 0.2 ml.) was drawn directly from the heart under light ether anesthesia and allowed to clot. The serum was obtained by centrifugation. All results were calculated in terms of phosphatase units, each unit equivalent to 1 mg. of phosphorus, as phosphate, liberated by 100 ml. of serum at pH 9.2 in one hour at 37 C. under the conditions of the experiment.

Histochemical Methods.—The procedure as described by Gomori[19] for the histochemical demonstration of alkaline phosphatase was used throughout with sodium glycerophosphate as substrate.[20] For controls the glycerophosphate was omitted from the incubation mixture. Incubation periods as short as one-half hour and as long as four hours were used as a means of better visualizing the amounts of enzyme present in the regenerating areas. Confirmatory histochemical detection of phosphatase was carried out by the method of Manheimer and Seligman.[21]

Administration of Phosphatase and of Phosphatase Inhibitors to Skin Wounds

Guinea pigs were placed on a scorbutogenic diet consisting of powdered purina® checkers, dried overnight at 100 C. Water was available at all times. After the animals had been restricted to the scorbutogenic diet for 16 days, skin wounds were made, one on each side of each animal, and blood samples were removed by heart puncture for determinations of serum alkaline phosphatase. An active preparation of calf intestinal phosphatase (1 ml. of which was capable of liberating 80,000 γ of phosphorus as phosphate from glycerophosphate in one hour at 37 C. and pH 9.2)[22] was applied in a sodium alginate base to the skin wounds twice daily, with the

16. The "checkers" are made by the Ralston Purina Company, St. Louis.

17. Shinowara, G. Y.; Jones, L. M., and Reinhart, H. L.: J. Biol. Chem. **142**:921, 1942.

18. Gould, B. S., and Shwachman, H.: Am. J. Physiol. **135**:485, 1942.

19. Gomori, G.: Am. J. Clin. Path. **16**:347, 1946.

20. Histochemical studies showed that the alkaline phosphatase present in healing rat-skin wounds was not highly specific. The enzyme was able to hydrolyze calcium hexose diphosphate, cocarboxylase, adenylic acid, cytidylic acid, guanylic acid, sodium nucleate, and depolymerized thymonucleic acid, as well as sodium glycerophosphate. Polymerized thymonucleic acid was not hydrolyzed by the wound phosphatase.

21. Manheimer, L. H., and Seligman, A. M.: J. Nat. Cancer Inst. **9**:427, 1948.

22. The purified intestinal phosphatase was supplied by Dr. Gerhard Schmidt of Tufts Medical School, Boston. Its activity may be compared with the activity of the serum of a normal guinea pig, 1 ml. of which can liberate only about 0.2 γ of phosphorus under similar conditions. Entire rat-skin wounds that have healed for six days and are high in phosphatase activity have a total phosphatase activity which is able to liberate about 39 γ of phosphorus in one hour at 37 C. and pH 9.4. Each application of calf intestinal phosphatase was able to liberate 4,000 γ of phosphorus under similar conditions.

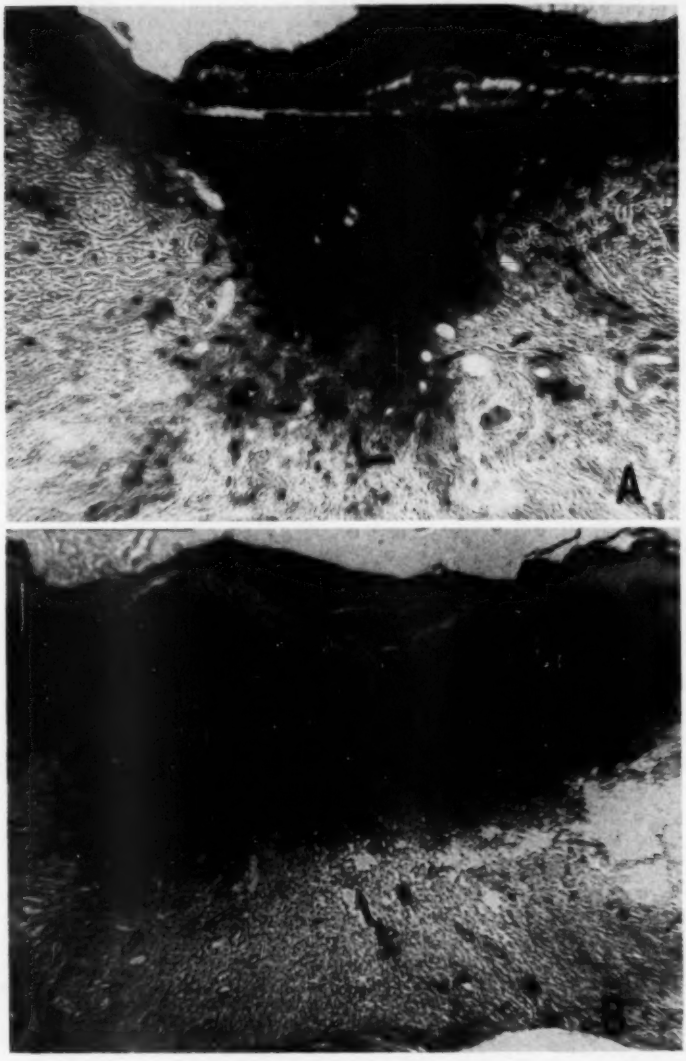

Fig. 1.—*A*, section of a 9-day-healed skin wound of a rat that had been fastened for several days, with the serum phosphatase falling to 23 units. It shows intense phosphatase activity. × 30.

B, section of a 9-day-healed skin wound of a rat maintained on a high-fat diet, with the serum phosphatase rising to 360 units. × 30.

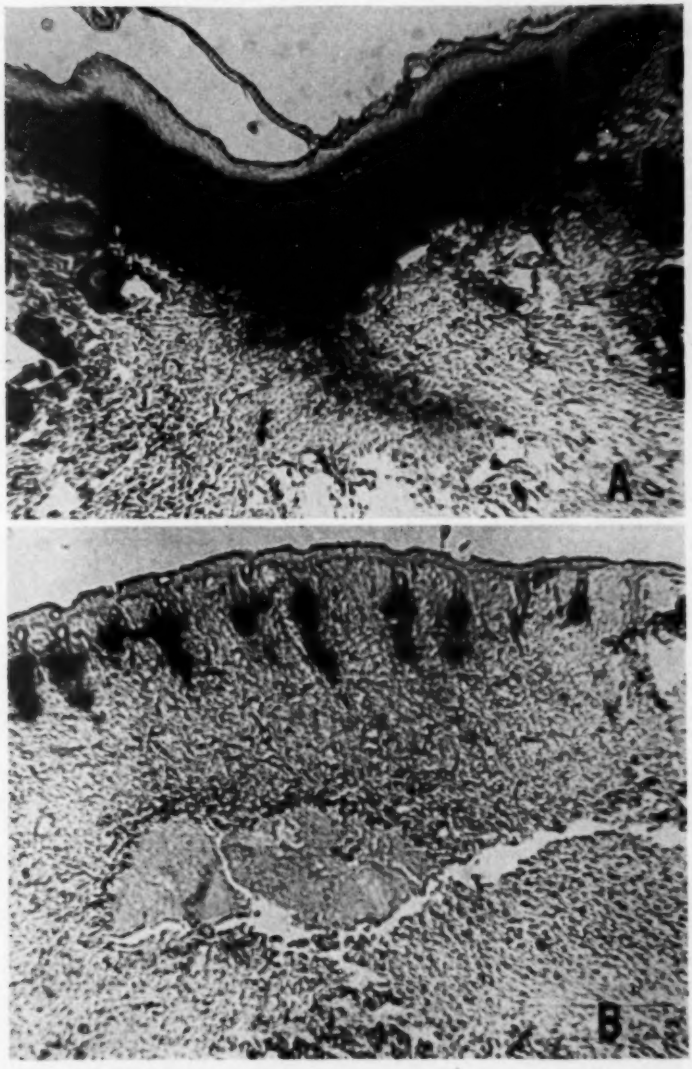

Fig. 2.—*A*, 9-day wound of the skin of a normal guinea pig, stained for phosphatase by the Gomori method. × 30.

B, 14-day scorbutic-guinea-pig skin wound which had been treated twice daily with a highly active phosphatase preparation in an alginate anchoring medium, then stained for phosphatase by the Gomori method. Only a very small amount of phosphatase is retained by the wound area, and no typical localization occurs. × 30.

alternate side of each animal serving as a control, for 14 days. At this time serum alkaline-phosphatase levels were again determined and the wounds excised, fixed in alcohol, sectioned at 7 μ, and stained histochemically for alkaline phosphatase.

Several other guinea pigs were maintained on a diet consisting of purina® checkers, with water available at all times and with 10 mg. of ascorbic acid being given daily throughout the experiment. These animals were considered the normal controls. They were wounded in the same fashion as the scorbutic animals. Their wounds were removed after 9 and 11 days, fixed in alcohol, sectioned at 7 μ, and stained histochemically for alkaline phosphatase. Representative sections of all wounds were stained with Delafield's hematoxylin-eosin and with Krichesky's [23] modification of Mallory's triple stain for connective tissue.

To demonstrate the effect of phosphatase inhibitors on the healing of skin wounds in the rat, 0.15 ml. of a 1% solution of phosphatase inhibitor or veronal buffer, both at pH 8, the latter acting as control, was injected three times daily for eight days into newly formed skin wounds. Injections were made as close to the surface of the wound as possible with a fine No. 37 needle. The wound on the left side of each rat received the veronal buffer, while that on the right side received either cysteine or thioglycolate as inhibitor. After eight days of such treatment, the wounds were excised, fixed in alcohol, embedded in paraffin, and sectioned at 7 μ. Sections of each wound were stained for glycerophosphatase activity, for connective tissue with Mallory's stain, and with hematoxylin-eosin.

RESULTS

Effects of Altering Serum Phosphatase Levels

The serum phosphatase levels ranged from 193 to 440 units in the rats on the high-fat diet. In the "normal" rats the range was from 47 to 92 units, and in the fasting animals, from 12 to 22 units.

Two-Day Wounds.—As reported by Fell and Danielli [2] the two-day-wound sections stained for phosphatase activity showed many darkly staining leucocytes and the entire wound was covered by a thick scab, sometimes loosened during fixation, composed of densely packed and heavily stained leucocytes. No phosphatase-active fibers were present, since no true connective tissue fibers were formed as yet. There was no difference in intensity of phosphatase between the wound sections from the low-serum-phosphatase and those from the high-serum-phosphatase rats.

Seven-Day Wounds.—Sections of wounds removed seven days after wound formation showed extensive epithelization and presence of much cellular material with strong phosphatase activity and young connective tissue also exhibiting marked phosphatase activity. It was not possible to distinguish between animals with very high levels of serum alkaline phosphatase and those with low levels. There appeared to be no marked differences in the sites and quantities of alkaline phosphatase in the wounds of the animals fed the various diets.

Nine-Day Wounds.—Sections of wounds excised nine days after wound formation and stained for alkaline phosphatase appeared very much like the seven-day wound sections. Epithelization was almost complete, and most of the scabs were gone. No distinction could be made between the sections taken from the animals fed the high-fat diet and having highly elevated serum-phosphatase levels and those from the fasted animals having low serum-phosphatase levels.

Gross observation failed to show any obvious difference in the over-all healing of any of the wounds.

Effects of Administering Phosphatase or Phosphatase Inhibitors to Skin Wounds

Phosphatase.—Guinea pigs were maintained on a scorbutogenic diet, and at the time of skin wounding the serum alkaline-phosphatase activity had fallen to the low

23. Krichesky, B.: Stain Technol. **6**:97, 1931.

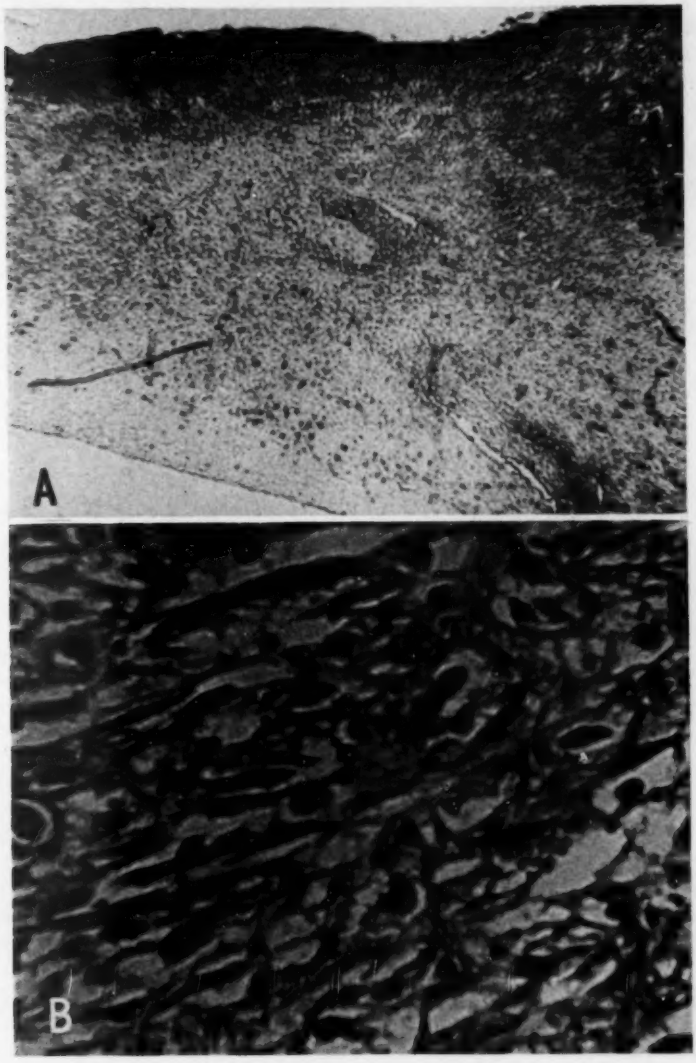

Fig. 3.—*A*, 8-day rat-skin wound which had three injections of cysteine daily. The Gomori phosphatase stain indicates marked inhibition of the phosphatase. × 30.

B, Mallory's connective tissue stain of a section shown in *A*, indicating an abundance of fibers in spite of low enzyme activity. × 450.

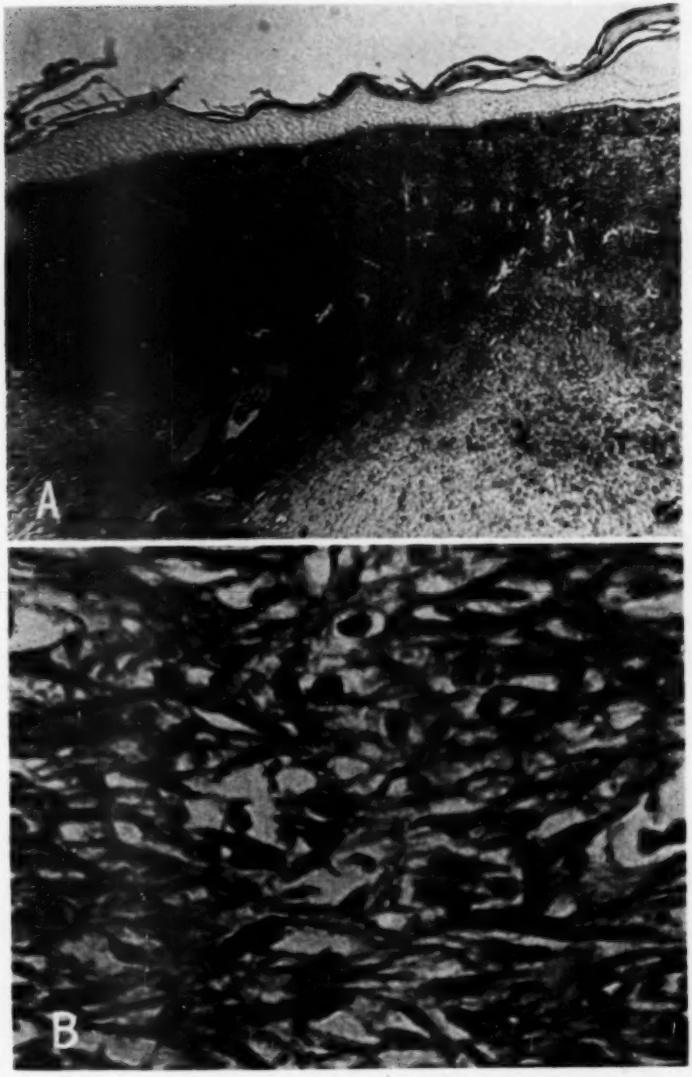

Fig. 4.—*A*, 8-day rat-skin wound stained by Gomori method. The wound received three injections of veronal buffer each day at pH 8.0 and acts as the control for wounds receiving injections of enzyme inhibitors. × 30.

B, section similar to *A* stained to show the abundance of fibers formed under conditions in which veronal buffer is injected. × 450.

levels of 0.5 to 3.3 units. Fourteen days after wounding the range was from 0.0 to 1.1 units. All of the animals showed gross symptoms of scurvy.

The alkaline-phosphatase activity of the 14-day skin wounds of scorbutic guinea pigs was almost negligible. The applications of alkaline phosphatase caused no fiber regeneration in the wound areas. Further, no appreciable amount of alkaline phosphatase was retained in the wound area, indicating that free phosphatase was rapidly removed from the wound.

In contrast to the skin wounds of the scorbutic animals, the 9- and 11-day-healed wounds of the normal guinea pigs showed abundant fiber regeneration and intense alkaline phosphatase activity.

Phosphatase Inhibitors.—The skin wounds of the rats began to heal in spite of the phosphatase inhibitors repeatedly injected into the wound areas. The wound receiving thioglycolate, however, appeared soft, and scab formation was retarded. Despite this, fibers formed, but appeared somewhat swollen. Glycerophosphatase activity was low in all the sections stained for enzyme activity.

The degree of fibrogenesis in the healing rat wounds receiving inhibitor compared favorably with that in 8-day untreated skin wounds.

COMMENT

That the serum alkaline-phosphatase level may be involved in wound healing was suggested as a result of studies made on scorbutic animals, in which little or no repair of connective tissue occurs and in which there is no concentration of alkaline phosphatase in an area of damaged tissue and serum phosphatase levels are strikingly low.

The results of the present experiments indicate that varying the serum alkaline-phosphatase levels in the rat to as low as 12 units and to as high as 440 units does not significantly alter the rate of healing of skin wounds, nor does it appear to alter the amount of alkaline phosphatase which is associated with the regenerating fibers of the wounds. This suggests that variation of the level of serum alkaline phosphatase does not affect the laying-down of connective tissue fibers significantly.

The fact that the low values of serum phosphatase reached in the fasting rat did not prevent proper connective tissue formation and did not appear to decrease the amount of alkaline phosphatase in the wound area may be explained on the basis that regenerating collagen fibers are perhaps able to adsorb only small amounts of phosphatase from the interstitial fluids. Once a saturation level of alkaline phosphatase has been attained on the fibers, an increase in the amount of alkaline phosphatase in the surrounding media results in no further adsorption of phosphatase. This is further suggested by the fact that introducing relatively massive amounts of phosphatase into scorbutic guinea pig wound areas where scanty fiber formation occurs did not result in sustained high levels in the wound. Correspondingly, decreases of serum phosphatase reaching fairly low levels are not accompanied by absence of phosphatase from the fibers. Gold and Gould [1] have shown that rat-tail collagen fibers precipitated in the presence of alkaline phosphatase will strongly adsorb phosphatase. In the scorbutic animal there is almost complete absence of fiber formation accompanied with low phosphatase activity in skin-wound areas. It is possible that the phosphatase is not present in large amounts because fibers are not present to adsorb the enzyme and wound phosphatase is rapidly eliminated.

While the low serum alkaline phosphatase obtained in rats by fasting is still somewhat higher in value than that determined in scorbutic guinea pigs and man, it is noteworthy that young partially scorbutic guinea pigs generally have higher serum alkaline-phosphatase levels than normal adult guinea pigs; yet connective tissue will be laid down in the adult guinea pigs but not in the scorbutic animals. Also, the difference in serum phosphatase between adult normal and adult scorbutic guinea pigs is relatively small, yet the former can synthesize new connective tissue, while the latter cannot. These facts suggest that the serum phosphatase level is not a prime factor in connective tissue formation.

Alkaline phosphatase applied to wound areas in scorbutic guinea pigs does not stimulate connective tissue fiber formation under the conditions of these experiments, and, furthermore, fiber formation proceeds in wounds of rat skin in the presence of powerful phosphatase inhibitors. While no evidence exists that the inhibitors persist in the wounds, histochemical examinations indicated that they had effectively inhibited the enzyme. These observations throw additional doubt on any special role of phosphatase in the synthesis of collagen fibers. In this respect, histochemical phosphatase studies of sections of the skin of 5-day-old rats are extremely interesting since, in spite of the fact that the animal is producing fibrous tissue very actively and in spite of the fact that the skin at this stage is relatively rich in phosphatase associated with the blood vessels and cells, little or none of the phosphatase is associated with the fibers. Furthermore, Robertson and associates [7] have shown that in the development of fibrous kidney capsules the newly formed collagen fibers are unaccompanied with high levels of alkaline-phosphatase activity.

Recent work of A. B. Novikoff [24] along lines previously carried out by Danielli [25] indicates that certain structures, notably the nucleus, have the ability to adsorb calcium phosphate and therefore give the impression that they contain phosphatase. This suggests the possibility that collagen fibers act in the same way. This possibility has been ruled out as the explanation for the strong staining observed about newly formed fibers by visualizing the phosphatase with the Manheimer-Seligman [21] histochemical method, which does not involve precipitation of insoluble calcium phosphate but rather precipitation of an easily visualized water-insoluble organic dye. This method of examining wounds gives a picture essentially like that described for the Gomori method and suggests that whatever the method of appearance on or about the fibers, it is in fact phosphatase which is being visualized.

CONCLUSIONS

The phosphatase activity which appears so dramatically associated with newly formed fibers in a healing wound is independent of marked changes in the phosphatase level of the blood.

Massive amounts of phosphatase applied to wounds of scorbutic animals where fiber formation is poor, and powerful phosphatase inhibitors injected into wounds in rats where fiber formation is rapid, are without influence, indicating a doubtful role for phosphatase in fiber formation.

The absence of phosphatase from areas of poor regeneration may be due to the absence of fibers which normally adsorb some phosphatase and concentrate it to the point where it can be visualized histochemically.

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24. Novikoff, A. B.: Personal communication to the authors.

25. Danielli, J. F.: J. Exper. Biol. **22**:110, 1946.

HEPATIC NECROSIS INDUCED BY DIETARY MEANS

III. The Effect of Various Dietary Modifications on the Liver Lipid Fractions and on the Development of Necrosis

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PREVIOUS studies by us [1] have shown that prior to and at the time of development of massive hepatic necrosis certain changes occurred in the liver lipids as indicated by both histological examination and chemical analysis. Total lipids, neutral fat, total fatty acids, and cholesteryl esters accumulated in the liver, primarily around the central vein areas, prior to the development of necrosis. On the other hand, the levels of free cholesterol and of phospholipid remained essentially unchanged during this period. The massively necrotic livers were found to differ from the non-necrotic livers obtained from animals killed after the same interval on the test diet, by a striking increase in free cholesterol and an equally striking decrease in phospholipid. The present paper describes an investigation designed to determine whether or not certain of these changes in liver composition observed during the prenecrotic phase and at the time of necrosis are inevitable features of the development of the hepatic lesion. The basal necrogenic diet was modified by the addition of supplements designed to prevent necrosis and/or alter the level of liver lipids.

EXPERIMENTAL PROCEDURE

Young male rats of the Sprague-Dawley strain, weight range 101 to 155 gm., were arranged in uniform groups of 20 and one of 10 (Group Q) having an average weight of 128 to 129 gm. The animals were offered daily approximately 8 gm. of the test diets, which were of the following basal composition: Fleischmann's active dried baker's yeast, 18%; corn starch, 71%; lard, 5%; a salt mixture,[1a] 3%; cod liver oil,[2] 2%, and a sugar-vitamin mixture, 1%. The latter supplied the following amounts of B vitamins per 8 gm. of food: thiamine hydrochloride, 0.02 mg.; riboflavin, 0.025 mg.; pyridoxine hydrochloride, 0.02 mg., and calcium pantothenate,

This investigation was financed by a grant from the National Research Council of Canada.

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From the Department of Pathological Chemistry, Faculty of Medicine, University of Western Ontario.

1. (*a*) Abell, M. R.; Beveridge, J. M. R., and Fisher, J. H.: Hepatic Necrosis Induced by Dietary Means: I. Structural Changes Occurring in the Liver During the Development of Necrosis, Arch. Path. **50**:1 (July) 1950. (*b*) Abell, M. R., and Beveridge, J. M. R.: Hepatic Necrosis Induced by Dietary Means: II. Biochemical Changes Occurring in the Liver During the Development of Necrosis, ibid. **50**:23 (July) 1950.

2. The preparation used was that of Mead Johnson & Company, containing 3,000 U. S. P. units of vitamin A and 400 U. S. P. units of vitamin D per gram.

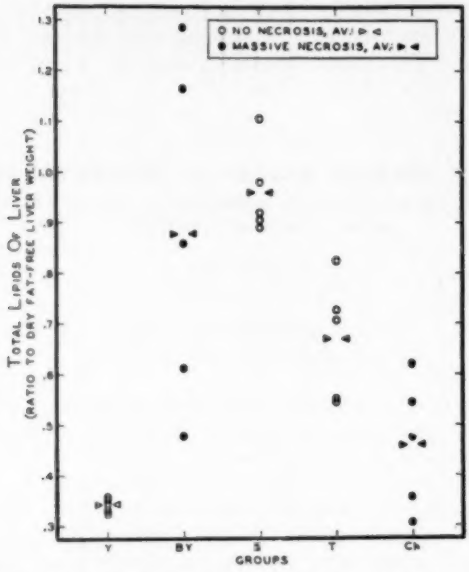

Chart 1.—Total lipid levels in the livers of rats fed diets of differing necrogenic and/or lipotropic properties.

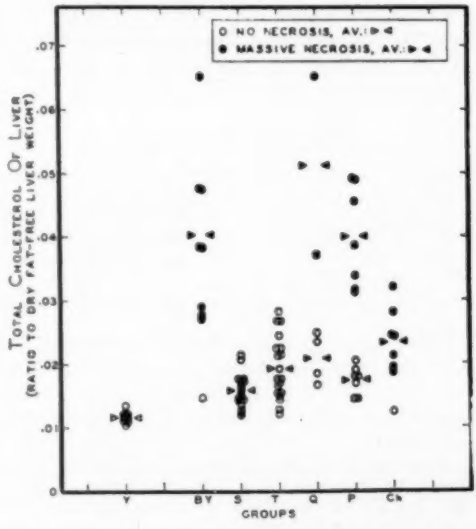

Chart 2.—Total cholesterol levels in the livers of rats fed diets of differing necrogenic and/or lipotropic properties.

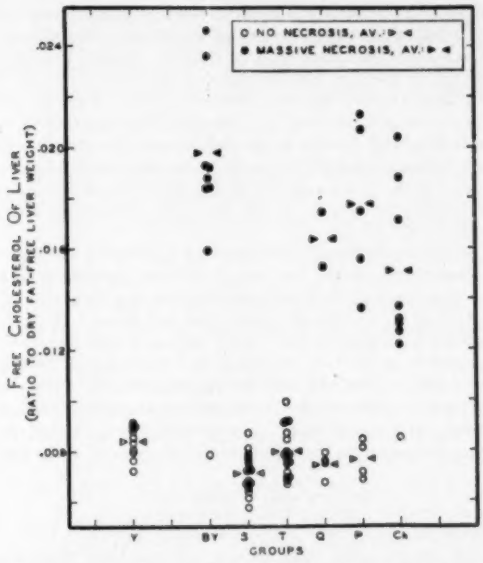

Chart 3.—Free cholesterol levels in the livers of rats fed diets of differing necrogenic and/or lipotropic properties.

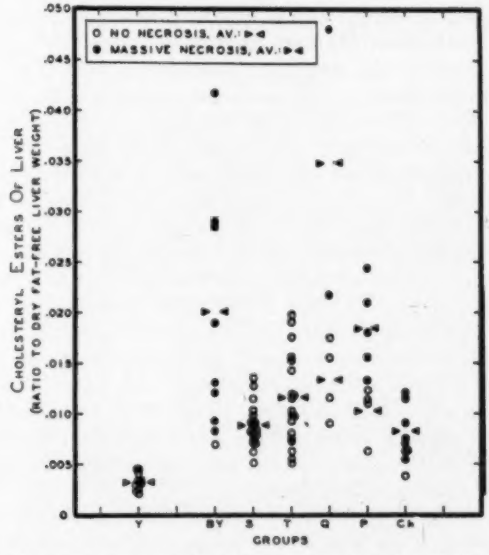

Chart 4.—Cholesteryl ester levels in the livers of rats fed diets of differing necrogenic and/or lipotropic properties.

0.1 mg. The supplements, which replaced equivalent amounts of starch, were as follows: Group BY, none; Group T, 2 mg. alpha-tocopherol (dissolved in ethyl laurate) daily by mouth; Group S, 0.5% l-cystine; Group Ch, 0.625% choline chloride; Group P, crystalline vitamins A (acetate) and D (calciferol) at levels of 1.38 mg. and 0.02 mg. respectively per 100 gm. of diet, these levels contributing approximately the same numbers of units of vitamins A and D as did the 2% level of cod liver oil incorporated into the basal diet. The cod liver oil was replaced by an equal amount of lard. Group Q received 5 mg. alpha-tocopherol quinone (dissolved in ethyl laurate) daily by mouth. Eleven animals (Group Y) were taken at random from the pool of rats and killed at the start of the test period. Their livers were treated as indicated below for the livers of the animals given the test diets, and the results were taken as representative of rats of this strain, age, and sex.

Autopsies were performed on all animals whether they died during the experimental period, were killed when moribund, or were killed at the end of the experiment (104 to 106 days). Sections were prepared from each of the three main lobes of the liver and, on the basis of a study of these, the presence or the absence of necrosis was ascertained. The remaining portions of the livers were analyzed for moisture, total lipids, and total and free cholesterol by procedures previously indicated.[1b] In the case of animals in which necrosis developed, only those livers showing massive necrosis were subjected to analysis. The few livers that manifested recurrent submassive necrosis or postnecrotic scarring were not subjected to analysis. With the exception just noted, total and free cholesterol determinations were carried out on all livers, whereas total lipid estimations were performed on five livers selected at random from Groups Y, BY, S, T, and Ch.

RESULTS AND COMMENT

The results from the lipid analyses are presented in Charts 1 to 4. An examination of Chart 1, which shows total lipid values, reveals that there has occurred a marked degree of fatty infiltration (compare Groups Y and BY) and that the inclusion of 0.625% choline chloride in the diet has partially but not completely prevented this accumulation of fat. Despite the obvious differences in total liver lipids between the two groups BY and Ch, the incidence of liver necrosis in both groups was exactly the same (95%). Furthermore, the liver lipids of the animals fed cystine (S), in none of which necrosis developed, were fully as elevated as those of the basal group (BY). These results constitute a confirmation and extension of the work of Himsworth and Glynn.[3] These workers fed a basal necrogenic diet to a group of rats and compared the amount of fatty acids plus unsaponifiable matter of the livers of animals in which necrosis developed with that of animals in which necrosis did not develop. They concluded: "Hepatic necrosis occurred at all levels of liver fat and afflicted animals showed the same proportions and amounts of liver fat as normal animals which had been taking the same diet for a similar length of time." The work reported in the present study shows that the total liver lipids can be strikingly varied without having any demonstrable effect on the development or the incidence of liver necrosis. The data shown in Charts 2 to 4, however, reveal that although total lipids may not change to any notable degree, significant changes do occur in the components of the liver lipids, a fact that has recently been reported by us.[1b]

In Chart 2, which shows total cholesterol levels, it can be appreciated readily that in all the livers showing necrosis there was a significant increase in total cholesterol. This fact is particularly evident when values from necrotic and nonnecrotic livers in the same group are compared.

The values for free cholesterol shown in Chart 3 reveal, as did our last study,[1b] the remarkable constancy of the levels of this substance in non-necrotic livers. The dramatic increase in free cholesterol is also obvious. That this change did not occur

3. Himsworth, H. P., and Glynn, L. E.: Biochem. J. **39**:267, 1945.

at the expense of cholesteryl ester is apparent from an examination of Chart 4, which shows the level of this liver component. Since the elevation of the level of free cholesterol occurred in all necrotic livers no matter what supplement had been given, it seems reasonable to conclude that this phenomenon is an inevitable feature of the pathological process under study.

Cholesteryl ester levels rose in all non-necrotic livers, and it is evident that a further rise occurred at the time of necrosis. The latter feature was not detected in our previous study,[1b] possibly because analyses were performed on the livers of animals that were killed at random during the test period when liver necrosis may have been developing in many of these animals. In such an event the cholesteryl ester might indeed be increased in these livers immediately prior to necrosis when histological signs of the latter were lacking. This would make it difficult to detect a rise in cholesteryl ester level at the time of necrosis under the conditions of our previous investigation.

It should be noted that choline prevented the increase in cholesteryl ester during the prenecrotic period in the one rat that escaped liver necrosis. There is in this group an increase at the time of necrosis—again the comparison is based on the value for the lone rat in which hepatic damage did not develop. Even with this increase in the cholesteryl ester fraction, the levels found in the necrotic livers from the group whose diet was supplemented with choline are, on the average, lower than those found for the groups whose diet was supplemented with cystine or alpha-tocopherol. In animals of the latter groups necrosis did not develop. One may therefore conclude that the level of cholesteryl ester is not of significance during the prenecrotic period. On the other hand, no matter what supplement is given, there is an increase in this fraction at the time of necrosis. The latter change, like that for the free cholesterol component, would appear to be an inevitable feature of liver necrosis.

SUMMARY

Groups of 20 male rats of the Sprague-Dawley strain, weight range 101 to 155 gm. and averaging 128 to 129 gm., were fed a basal necrogenic diet, low in the sulfur-containing amino acids and in alpha-tocopherol, to which were added supplements designed to prevent liver necrosis and/or alter the level of liver lipids. These supplements consisted of cystine, choline chloride, alpha-tocopherol, alpha-tocopherol quinone, and crystalline vitamins A and D in place of cod liver oil. The feeding period was terminated after 104 to 106 days.

The livers were analyzed for moisture, total lipids, and total and free cholesterol. The results showed that fatty infiltration occurred during the prenecrotic period and that choline chloride at a level of 0.625% of the diet only partially prevented this accumulation of fat in the liver. There was no correlation between liver lipid levels and the development of massive liver necrosis.

Free cholesterol levels remained relatively constant in all non-necrotic livers but increased markedly at the time of necrosis. The cholesteryl ester fraction was found to be increased in the non-necrotic livers, and a further rise was noted at the time of development of liver necrosis. The changes in free and esterified cholesterol, at the time of necrosis, occurred in all groups and therefore may be considered to be inevitable accompaniments of the process under study.

STUDIES ON HEPATIC NECROSIS INDUCED BY DIETARY MEANS

IV. Conditions Affecting the Production and Prevention of Massive Liver Necrosis

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THE OBSERVATION by György and Goldblatt [1] that the feeding of diets to rats designed to produce cirrhosis of the liver actually caused death due to necrosis of this organ initiated intensive research by many workers to ascertain the exact cause or causes of this lethal lesion. In brief review, Daft, Sebrell, and Lillie [2] found that methionine or choline in adequate quantities prevented fatty infiltration and cirrhosis, whereas cystine exerted no prophylactic action. Both the sulfur-containing amino acids prevented the development of liver necrosis, but choline was without any protective effect. On the basis of these findings, they postulated that the necrosis and cirrhosis described by György and Goldblatt were two separate and distinct entities, a conclusion that was supported subsequently by Himsworth and Glynn [3] and other workers. Many competent investigators [4] attempted to produce liver necrosis by using diets similarly deficient in protein and in the sulfur-containing amino acids but failed to obtain comparable results. This inconsistency led us, in 1948, to make a critical comparison of the diets used by the different groups, and it was noted that all rations that were notably successful in producing necrosis of the liver contained cod liver oil as a source of vitamins A and D; the fat component was relatively low in alpha-tocopherol and, furthermore, alpha-tocopherol was not included in the vitamin supplements. These points were taken into consideration, and it was found that the lesion could be readily and consistently produced and with

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The vitamin B_{12} used in this study was donated by E. R. Squibb & Sons of Canada Limited. Montreal, Canada.

1. György, P., and Goldblatt, H.: J. Exper. Med. **70**:185, 1939.

2. Daft, F. S.; Sebrell, W. H., and Lillie, R. D.: Proc. Soc. Exper. Biol. & Med. **50**:1, 1942.

3. Himsworth, H. P., and Glynn, L. E.: Clin. Sc. **5**:93 and 133, 1944; Proc. Roy. Soc. Med. **38**:101, 1944; Lancet **1**:457, 1944.

4. Irving, J. T., and Schwarz, H. M.: Clin. Proc. **5**:14, 1946. Neuberger, A., and Webster, T. A.: Biochem. J. **41**:449, 1947. Sellers, E. A.; Lucas, C. C., and Best, C. H.: Unpublished data, cited by Sellers, E. A.: Canad. M. A. J. **59**:403, 1948. Radhakrishna Rao, M. V.: Nature, London **161**:446, 1948. Hall, C. A., and Drill, V. A.: Proc. Soc. Exper. Biol. & Med. **70**:202, 1949.

an incidence higher than any previously published (100%).[5] The prophylactic role of alpha-tocopherol in hepatic necrosis has been recently unequivocally established by Himsworth and Lindan [6] and by György and Goldblatt.[7a] Similar results have been obtained in our laboratory.[7b]

With regard to this phase of the work, in 1944, Schwarz,[8] working in Germany, described a type of liver necrosis that could be prevented by the inclusion of alpha-tocopherol in the ration. However, it seems apparent that the etiology of the lesion due to the feeding of alkali-treated casein is somewhat different from that described in this paper, a point that will be amplified later. The following experiments were designed to elucidate further the conditions affecting the production and the prevention of massive liver necrosis.

EXPERIMENTAL PROCEDURE

The following experiments were carried out to ascertain the influence, on the development of liver necrosis, of age, of sex, and of the following dietary supplements: cod liver oil, alpha-tocopherol quinone, synthetic vitamin K (menadione), l-tyrosine, l-ascorbic acid, i-inositol, choline, cholesterol, theophylline, xanthine, uric acid, propylthiouracil, desiccated thyroid, brewer's yeast, heat-killed baker's yeast, sulfaguanidine, aureomycin, and vitamin B_{12}. Diets containing alpha-tocopherol and l-cystine were also included. The exact amounts and details regarding these supplements and the rationale for testing their effects are presented under the section headed "Results and Comment."

All rats were of the Sprague-Dawley strain and, except for one group of females, were males. They were housed in individual cages and were offered daily approximately 8 gm. of the test diets, which were of the following basal composition: Fleischmann's active dried baker's yeast, 18%; corn starch, 71%; lard, 5%; a salt mixture,[9] 3%; cod liver oil,[10] 2%, and a sugar-vitamin mixture, 1%. The latter supplied the following amounts of B vitamins per 8 gm. of food: thiamine hydrochloride, 0.02 mg.; riboflavin, 0.025 mg.; pyridoxine hydrochloride, 0.02 mg., and calcium pantothenate, 0.1 mg. With the exception of the animals of Group A (adult males), which were allowed to live out their life span, the experiments were terminated at 104 to 106 days.

After death of the rats due to liver necrosis or after they were killed, small blocks of tissue were taken from each of the three lobes of the liver, and sections from each block were stained with hematoxylin and eosin. Although it was usually possible to detect the presence or the absence of necrosis by gross inspection of the liver, the microscopic examination was always performed.

RESULTS AND COMMENT

The calculation of the final incidence of liver necrosis presented a difficult problem in a few groups where death of a number of the animals occurred owing to causes other than liver necrosis. In preparing the following tables we arbitrarily decided to eliminate from consideration all animals that died of causes other than liver necrosis if they died prior to the time at which the first death due to hepatic damage occurred in the remainder of the group.

5. Abell, M. R., and Beveridge, J. M. R.: Canad. J. Res. Sect. E **27**:316, 1949..

6. Himsworth, H. P., and Lindan, O.: Nature, London **163**:30, 1949.

7. (*a*) György, P., and Goldblatt, H.: J. Exper. Med. **89**:245, 1949. (*b*) Abell, M. R., and Beveridge, J. M. R.: Am. J. Path. **26**:713, 1950.

8. Schwarz, K. C.: Ztschr. f. physiol. Chem. **281**:101 and 109, 1944.

9. Abell, M. R.; Beveridge, J. M. R., and Fisher, J. H.: Arch. Path. **50**:1 (July) 1950.

10. The preparation used was that of Mead Johnson & Company, containing 3,000 U. S. P. units of vitamin A and 400 U. S. P. units of vitamin D per gram.

Effect of Initial Weight and of Sex on the Development of Liver Necrosis.—The marked effect of the initial weight and the sex of rats on the development of liver necrosis is obvious from an examination of Table 1. Although the final incidence of necrosis is essentially the same in all the male animals of differing initial weights, the intervals required for liver damage to occur were widely divergent. Thus for animals of initial weights 53, 129, and 235 gm., the average number of days at which death due to hepatic necrosis occurred was respectively, 38, 58, and 205. This result indicates either that normal rats have a store of a substance that prevents the development of liver damage under these conditions or that the demand for the missing factors, the sulfur-containing amino acids and alpha-tocopherol, decreases with increasing age.

Lindan and Himsworth [11] have recently shown that supplementing the diet of rats with cystine or alpha-tocopherol prior to feeding the animals a necrogenic diet retards the appearance of hepatic necrosis. However, the possibility that the demand for those substances is increased in the younger age groups cannot be discarded. These workers also demonstrated that older rats are more resistant to the development of hepatic necrosis. The report of Handler and Follis [12] concerning the influ-

TABLE 1.—*Effect of Initial Weight and of Sex on the Development of Liver Necrosis*

				Rats That Died of Necrosis or Were Killed When Moribund		Rats Killed at 105 Days		Total Incidence of Necrosis*	
Group	Rats	Av. Initial Wt., Gm.	Sex	Rats	Days on Diet (Av. Range)	No. Showing Signs of Necrosis	No. Showing No Signs of Necrosis	No. of Rats	Per Cent
W	20	53	M	19	38 (25-61)	..	..	19/20	95
Y	20	129	M	16	58 (35-97)	3	1	19/20	95
YF	20	129	F	16	79 (48-108)	..	4	16/20	80
A	20	235	M	18	205 (161-241)	..	..	18/19	95

* Rats that died of causes other than that of liver necrosis were eliminated from consideration in the calculation of the total incidence of necrosis if they died prior to the time at which the first death due to hepatic damage occurred in the remainder of the group. For example: Three animals died of intercurrent infections during the experiment, one from Group W at 33 days and two from Group A at 119 and 200 days. In accordance with the foregoing convention, only the rat that died at 119 days has been eliminated in calculating the total incidence of liver necrosis.

ence of age on hepatic response to choline and cystine deficiency in the rat is difficult to interpret. They concluded that necrosis occurred more quickly in younger rats. However, they utilized a basal diet containing alpha-tocopherol in the vitamin supplements, and a further significant amount would be supplied in the form of cottonseed oil, which was fed at the 10% level. Finally, it has been our experience that massive necrosis does not readily occur on a diet containing 5% casein, the level utilized by Handler and Follis. These considerations pose the question whether or not the lesion observed by these workers was in fact identical with that discussed in this paper, i. e., massive liver necrosis.

A comparison of the data in Table 1 from Groups Y and YF, males and females, respectively, reveals that although the total incidence of necrosis is almost the same (95% vs. 80%), the average length of time required for death due to liver damage to ensue is markedly greater in the case of the females (58 vs. 79 days). This difference has been shown to be highly significant ($P = <0.01$). Two possible

11. Lindan, O., and Himsworth, H. P.: Brit. J. Exper. Path. **31**:651, 1950.
12. Handler, P., and Follis, R. H., Jr.: Proc. Soc. Exper. Biol. & Med. **75**:567, 1950.

explanations for the sex differences may be suggested. The difference in the response of the two sexes obviously may reside in their divergent hormonal environment; or, differences may exist in the protein requirements of the two sexes, a possibility for which evidence was adduced previously by Beveridge.[13]

It is of interest to note that Weichselbaum,[14] who probably first produced the type of massive liver necrosis being studied here, although it was not described as such, reported that the mortality with his experimental diets was much lower in the female rats. This finding was undoubtedly due to the longer time required to produce the lesion in this sex and to the fact that the experimental period was only a little over six weeks—not definitely specified. In the light of the clear-cut difference between the responses of the sexes, it appears to be essential to take this factor into account, a precaution that has not always been observed by workers in this field.[11]

Effect of Cod Liver Oil and of Crystalline Vitamins A and D on the Development of Liver Necrosis.—The importance of dietary cod liver oil on the develop-

TABLE 2.—*Effect of Various Dietary Supplements on the Development of Liver Necrosis*

Group	Rats	Av. Initial Wt., Gm.	Dietary Supplement *	Rats That Died of Necrosis or Were Killed When Moribund		Rats Killed at 104-106 Days		Total Incidence of Necrosis (Corrected)†	
				Rats	Days on Diet (Av. Range)	No. Showing Signs of Necrosis	No. Showing No Signs of Necrosis	No. of Rats	Per Cent
Y	20	129	None	16	58 (35-97)	3	1	19/20	95
P	20	129	Cryst. Vit. A and D and 2% lard in place of 2% cod liver oil	10	74 (51-98)	1	9	11/20	55
C	10	129	5% cod liver oil	10	46 (20-84)	..	..	10/10	100
T	20	128	2 mg. α-tocopherol/rat/day	0	.....	0	19	0/19	0‡
Q	10	129	5 mg. α-tocopherol quinone/rat/day	3	85 (55-101)	1	4	4/10	40‡
K	20	128	0.025% menadione	16	71 (49-102)	3	1	19/20	95
Ch	20	129	0.625% choline chloride	17	60 (42-103)	2	1	19/20	95
S	20	129	0.5% cystine	0	.....	0	20	0/20	0
Ty	10	128	1% tyrosine	10	50 (39-81)	..	..	10/10	100

* The supplements for Groups Ty, Ch, K and S replaced equivalent amounts of corn starch in the diet. In Groups T and Q the alpha-tocopheral and alpha-tocopherol quinone were dissolved in ethyl laurate and given daily by mouth just before the daily allotment of food was placed in the cage. The diet fed to Group P contained amounts of crystalline vitamins A (acetate) and D (calciferol) equivalent to the amounts of those substances in 2% cod liver oil, the latter being replaced by an equal amount of lard. The diet fed to Group C was modified by the addition of 5% cod liver oil in place of a like amount of lard, thus making a total of 7% cod liver oil in the ration.

† See footnote to Table 1.

‡ Three animals died of intercurrent infections during this experiment and are not accounted for in this table. Two were from Group Q and died at 79 and 88 days; one was from Group T and died at 20 days.

ment of liver necrosis has previously been reported by us,[5] but at that time a cod liver oil concentrate was used to substitute for the liver oil product. It was believed that if the results could be confirmed by using crystalline preparations of vitamins A and D in place of the cod liver oil that the results would have greater significance. The dietary conditions and results are shown in Table 2.

Group Y received the basal diet which contained 2% cod liver oil; Group P was fed a diet containing amounts of crystalline vitamins A (acetate) and D (calciferol) equivalent to the amounts of those substances in 2% cod liver oil, the latter

13. Beveridge, J. M. R.: J. Fish. Res. Bd. Canada **7**:35, 1947.
14. Weichselbaum, T. E.: Quart. J. Exper. Physiol. **25**:363, 1935.

being replaced by an equal amount of lard; Group C received a diet modified by the addition of 5% cod liver oil in place of a similar amount of lard, thus making a total of 7% cod liver oil in this ration.

The effect of eliminating the cod liver oil from the diet is obvious from a comparison of the incidence of liver necrosis in Groups Y and P (95% vs. 55%). The deleterious effect of increasing the cod liver oil component from 2% to 7% cannot be demonstrated by a comparison of the incidence of necrosis on Groups Y and C since on the former diet, which contained only 2% cod liver oil, the incidence was 95%. However, an examination of the average length of time at which liver necrosis caused death makes it apparent that cod liver oil increases the necrogenic quality of the diet. The average length of time at which death from hepatic necrosis occurred was 58 days for Group Y and only 46 days for Group C. The elimination of cod liver oil, besides decreasing the incidence of necrosis, markedly increased the length of time required to induce hepatic damage (74 vs. 58 days, $P = <0.01$). These results completely substantiate our previous finding [5] that cod liver oil augments the production of liver necrosis. It is possible that this necrogenic effect of cod liver oil may be due to its antagonistic effect on the action of alpha-tocopherol.[15]

Effect of Alpha-Tocopherol, Alpha-Tocopherol Quinone, and of Synthetic Vitamin K (Menadione) on the Development of Liver Necrosis.—In 1945, Woolley [16] reported that alpha-tocopherol quinone when administered to pregnant mice caused hemorrhage into the reproductive tract and resorption of fetuses. This process could not be prevented by alpha-tocopherol but menadione (2-methyl-1,4-naphthoquinone) was found to have a preventative action. It seemed worth while to ascertain whether or not a similar interrelationship existed amongst these compounds with regard to the manifestation of alpha-tocopherol deficiency being investigated here.

Groups T and Q received respectively 2 mg. of alpha-tocopherol and 5 mg. of alpha-tocopherol quinone [17] per rat per day. These substances were dissolved in ethyl laurate and administered by mouth via a dropper prior to placing the daily allotment of food in the cage. Group K received a supplement of 2 mg. of menadione per 8 gm. of diet.

An examination of Table 2 shows that alpha-tocopherol was completely successful in preventing the liver lesion. This confirms the work of Himsworth and Lindan [6] and György and Goldblatt.[7a] It is obvious, however, that the alpha-tocopherol quinone did not act as a metabolic antagonist for alpha-tocopherol, and indeed it actually showed some preventative action since the average time at which necrosis developed was 85 days, compared with 58 days for the basal diet fed to Group Y, and the incidence was respectively 40% and 95%. The incidence of liver damage in Group K was exactly the same as that in the basal group, but the average length of time at which necrosis developed was greater in the former group (71 vs. 58 days), indicating that vitamin K has an ameliorating action on the development of necrosis. This difference was shown to be statistically significant ($P = <0.01$).

15. Mackenzie, C. G.; Mackenzie, J. B., and McCollum, E. V.: J. Nutrition **21**:225, 1941.

16. Woolley, D. W.: J. Biol. Chem. **159**:59, 1945.

17. The alpha-tocopherol quinone was prepared from alpha-tocopherol by the method of P. Karrer and A. Geiger (Helvet. Chem. Acta **23**:455, 1940). Analysis of the product by the procedure of H. Kaunitz and J. J. Beaver (J. Biol. Chem. **156**:653, 1944) showed the oxidation of alpha-tocopherol to be complete (98%).

The interrelationships amongst alpha-tocopherol, alpha-tocopherol quinone, and menadione in the manifestation of alpha-tocopherol deficiency studied in this paper are not the same as those found by Woolley [16] in his investigation on fertility in mice. On the other hand, it is interesting to note that synthetic vitamin K lessened the necrogenic quality of the basal diet. Work is now being carried out to obtain further information on this point.

Effect of Lipotropic and Antilipotropic Substances—Inositol, Choline, Cystine, and Cholesterol—on the Development of Liver Necrosis.—Previous work by us [18] demonstrated that when rats are fed a basal necrogenic diet, certain changes occur in the liver lipid fractions prior to and at the time of necrosis. This finding, coupled with the fact that cystine, an antilipotropic substance, prevents dietetic liver necrosis, indicated the advisability of performing a study utilizing as supplements various lipotropic and antilipotropic substances. Liver lipid analyses have been performed on these and other groups and have been reported in the preceding paper.[19]

An added reason for testing the effect of inositol, one of the factors chosen to be tested, was the fact that reports have been published linking closely the metabolism of this compound to that of alpha-tocopherol. Dam [20] has indicated that the symptoms of vitamin E deficiency in the chick can be prevented by inositol, and Milhorat and Bartels [21] have presented evidence suggesting that inositol and alpha-tocopherol form a complex in the stomach before being absorbed.

Supplements of 0.5% inositol, 0.625% choline chloride, 0.5% cystine, and 1% cholesterol were added to the food for Groups I, Ch, S, and CTL respectively. These substances replaced similar amounts of corn starch. Groups Ch and S were placed on the experimental regimen a few weeks prior to Groups I and CTL and differed also in that there were 20 animals in each group whose average initial weight was 128 to 129 gm., in contrast to 10 animals per group of an average initial weight of 51 gm. for the latter groups. In each case, therefore, comparisons must be made with the appropriate group fed the basal diet, i. e., Group Y in Table 2 for the heavier animals and Group W in Table 3 for the lighter animals.

An examination of the data in Table 2 shows the well-known preventative action of cystine. The supplement of choline chloride exerted no effect on the development of hepatic damage. Earle and Victor [22] and Himsworth and Glynn [23] concluded from their studies that choline chloride increased the necrogenic quality of diets low in protein. Our results indicate that it has no demonstrable effect.

The cholesterol or inositol supplementation of the basal diet did not materially affect the necrogenic quality of the diet since the incidence of necrosis and the average length of time required for the occurrence of death due to liver damage was essentially unchanged (Table 3; compare Groups CTL and I with Group W). It may be concluded, therefore, that with the exception of cystine none of these lipotropic or antilipotropic agents exerted any detectable effect on the production of liver necrosis.

18. Abell, M. R., and Beveridge, J. M. R.: Arch. Path. **50**:23 (July) 1950.
19. Abell, M. R., and Beveridge, J. M. R.: Arch. Path., this issue, p. 423.
20. Dam, H.: J. Nutrition **27**:193, 1944.
21. Milhorat, A. T., and Bartels, W. E.: Science **101**:93, 1945.
22. Earle, D. P., and Victor, J.: J. Exper. Med. **39**:817, 1942.
23. Himsworth, H. P., and Glynn, L. E.: Clin. Sc. **5**:133, 1944.

Effect of Purines on the Development of Liver Necrosis.—Attention has previously been drawn by György and Rose [24] to the similarity in the compounds that aid in the prevention of alloxan diabetes and dietary liver necrosis respectively. Recently Griffiths [25] showed that by feeding rabbits a diet low in methionine and cystine blood glutathione levels could be greatly reduced and a diabetic condition of several days' duration could be induced by the administration of uric acid. The latter compound, which is closely related structurally to alloxan, has a qualitatively similar diabetogenic effect but by contrast is a substance naturally occurring in the body. According to the reasoning set forth by György and Rose,[24] it was believed quite likely that uric acid might have a necrogenic action.

The report by Hove [26] on the protective effect of alpha-tocopherol, methionine, theophylline, and xanthine against the toxic action of carbon tetrachloride in rats fed diets deficient in protein prompted us to test the effect of these other purine derivatives, theophylline and xanthine, on the liver lesion under investigation in this

TABLE 3.—*Effect of Various Dietary Supplements and Modifications on the Development of Liver Necrosis*

Group	Rats	Av. Initial Wt., Gm.	Dietary Supplement or Modification	Rats That Died of Necrosis or Were Killed When Moribund		Rats Killed at 104-106 Days		Total Incidence of Necrosis (Corrected) *	
				Rats	Days of Diet (Av. Range)	No. Showing Signs of Necrosis	No. Showing No Signs of Necrosis	No. of Rats	Per Cent
W	10	51	None	9	39 (23-48)	..	..	9/10	90
I	10	51	0.5% inositol	9	35 (28-54)	..	..	9/10	90
CTL	10	51	1% cholesterol	10	37 (29-57)	..	..	10/10	100
U	10	53	1% uric acid	9	39 (32-47)	..	..	9/10	90
TH	10	56	0.2% theophylline	6	36 (28-45)	..	..	6/ 9	67
X	10	56	0.2% xanthine	9	44 (30-55)	..	..	9/10	90
VC	10	51	0.625% ascorbic acid	8	49 (25-80)	..	..	8/10	80
HY	10	50	Heated yeast †	10	39 (32-48)	..	..	10/10	100
BrY	10	51	Brewer's yeast ‡	9	48 (21-66)	..	1	9/10	90
PR	10	56	0.3% propylthiouracil	0	.....	..	..	0	0 ‡
DT	10	56	0.3% desiccated thyroid	7	20 (16-24)	..	..	7/ 8	88
SG	10	56	1% sulfaguanidine	3	96 (92-104)	..	1	3/ 4	75
VM	10	56	Modified vitamin mixture §	10	41 (31-52)	..	..	10/10	100

* See footnote to Table 1.

† Fleischmann's active dried baker's yeast was heated in an open pan in an oven at 60 C. for a period of one hour.

‡ U. S. P. grade obtained from Nutritional Biochemicals Corporation, Cleveland.

§ The following vitamin mixture replaced that used in the basal diet: thiamine hydrochloride, 0.050 gm.; pyridoxine hydrochloride, 0.020 gm.; calcium pantothenate, 0.100 gm.; nicotinic acid, 0.100 gm.; paraaminobenzoic acid, 1.000 gm.; folic acid, 0.005 gm.; biotin, 0.003 gm.; and vitamin B_{12}, 0.00006 gm., and sufficient powdered sugar to make the mixture up to 100 gm. This vitamin mixture was fed at a 1% level.

‡ The animals in this group died after an average of 47 (44 to 53) days on the test diet and showed no evidence of necrosis at death. The cause of death could not be ascertained.

paper. It is also of interest to note that Schwarz [8] found that xanthine prevented liver necrosis in rats fed alkali-treated casein.

A comparison of the results from Groups U (uric acid at 1% level), TH (theophylline at 0.2% level), and X (xanthine at 0.2% level) with those from Group W fed the basal diet (Table 3) shows that uric acid did not increase the necrogenic quality of the basal diet and that neither theophylline nor xanthine had any pro-

24. György, P., and Rose, C. S.: Science **108**:716, 1948.

25. Griffiths, M.: J. Biol. Chem. **172**:716, 1948.

26. Hove, E. L.: Arch. Biochem. **17**:467, 1948.

phylactic action on the development of liver necrosis. The ineffectiveness of xanthine indicates that the type of necrosis caused by alkali-treated casein is different from that discussed in this report, a conclusion also reached by Schwarz.[27]

Effect of Tyrosine and of Ascorbic Acid on the Development of Liver Necrosis.—In 1945, Glynn, Himsworth, and Neuberger[28] noted the presence of homogentisic acid in the urine of rats fed necrogenic diets deficient in methionine and cystine. This observation, together with the fact that alcaptonuria, produced experimentally by feeding excessive amounts of the aromatic amino acids, can be prevented by the addition of ascorbic acid,[29] drew attention to the possible implication of a disturbance in the metabolism of ascorbic acid and the aromatic amino acids, tyrosine and phenylalanine, in the etiology of dietetic liver necrosis. If such was the case, one could reasonably assume that the ability of the rat to synthesize ascorbic acid had been decreased. The fact that dietary protein, and specifically methionine and cystine, increased ascorbic acid synthesis by the rat[30] was taken as lending weight to the possibility outlined here. It was reasoned that if the foregoing assumptions were correct, the factor causing liver necrosis might be produced in consequence of the derangement in the metabolism of ascorbic acid and/or of the aromatic amino acids. In such an event the tyrosine supplementation of the basal diet should increase the necrogenic quality of the diet whereas ascorbic acid supplementation should decrease it.

The supplement l-tyrosine (1%) given to Group TY did not significantly affect the necrogenic quality of the basal diet (Group Y, Table 2). It may be noted that on the former ration all 10 rats died of massive liver necrosis at an average time of 56 days and on the basal diet 16 of 20 died at an average time of 58 days and one survived the experimental period, showing no signs of hepatic necrosis.

The effect of supplementation with 0.625% ascorbic acid was determined, using weanling rats, and the results are shown in Table 3 (Groups VC and W). Although there was no difference in the incidence of liver necrosis, there was an increase in the average length of time required for death to occur in the group whose diet was supplemented with ascorbic acid (49 vs. 39 days). The difference could not be shown to be statistically significant ($P = >0.05$). Although the results of feeding ascorbic acid and additional tyrosine might be interpreted as lending some support to our hypothesis, they are not sufficiently clear-cut to substantiate it by any means. It must be concluded, therefore, that a disturbance of ascorbic acid synthesis and a consequent derangement of the metabolism of the aromatic amino acids are probably not integrally involved in dietary liver necrosis.

Effect of Modifying the Yeast Component of the Basal Diet on the Development of Liver Necrosis.—In previous work by us, both published[5] and unpublished, liver necrosis has always occurred more readily on diets containing yeast as the protein moiety than on those in which the latter is supplied in the form of casein. György and associates[7a] have also commented on this fact and have drawn attention to the varying necrogenic properties of different yeasts.[31] They postulated that these

27. Schwarz, K.: Ann. New York Acad. Sc. **52**:225, 1949.
28. Glynn, L. E.; Himsworth, H. P., and Neuberger, A.: Brit. J. Exper. Path. **26**:326, 1945.
29. Sealock, R. R., and Silberstein, H. E.: Science **90**:517, 1939.
30. Roberts, E., and Speigel, J. C.: J. Biol. Chem. **171**:9, 1947.
31. (*a*) György, P.; Rose, C. S.; Tomarelli, R. M., and Goldblatt, H.: J. Nutrition **41**:265, 1950. (*b*) György, P., and Goldblatt, H.: Proc. Soc. Exper. Biol. & Med. **76**:124, 1951.

foods may differ in their content of protective substances or contain variable amounts of a toxic factor, or may differ in their ability to affect the production of such factors in the gastrointestinal tract. Although extensive amino acid analyses have been performed by Lindan and Work [32] on two brands of yeast of differing necrogenicity, the cause for this variability has yet to be ascertained.

In all previous diets containing yeast, we have utilized a preparation of baker's active dried yeast (Fleischmann's). The fact that certain live yeasts not only do not contribute certain of the B vitamins to the animal organism but may actually deprive it of these substances was demonstrated by Ness, Price, and Parsons.[33] In order to eliminate the possibility that this process accounted for the necrogenic property of the live yeast in the basal diet, a sample was heated for one hour at 60 C. before being incorporated into the ration for Group HY. It is obvious that this treatment had no effect on the outcome of the experiment (Table 3, Group HY vs. Group W), a result that is in agreement with the results of a similar experiment which has been reported recently by György and Goldblatt.[31b]

These workers and their colleagues have drawn attention to the low incidence of liver necrosis that was obtained by them utilizing brewer's yeast,[34] in contrast to the high incidence when baker's yeast [35] was employed.[31] Prior to the publication of these papers, we had utilized brewer's yeast [36] in place of baker's yeast. The results, shown in Table 3 (Groups BrY and W), reveal no change in the incidence of necrosis, but it is interesting to note that a longer time was required for animals to die of liver damage in the group fed brewer's yeast (48 days for Group BrY, compared with 39 days for Group W). It may be concluded, therefore, that the necrogenic property of the brewer's yeast is slightly less than that of the baker's yeast. Dr. György suggested that we compare the necrogenicity of the sample of brewer's yeast used by him with one of the brands currently being used by us. This comparison has just been completed at Queen's University, where the investigation of this problem is being continued under the direction of one of us (J. M. R. B.). Under the same experimental conditions described in this article U. S. P.-grade brewer's yeast [36] was compared with the sample of brewer's yeast [34] forwarded by Dr. György. The incidence of necrosis was 100% and 80% respectively and the average length of time required for the occurrence of death due to necrosis was 49 and 64 days respectively. These results confirm those of György and associates.[31] It is of interest to note that the results of feeding the U. S. P.-grade brewer's yeast at different times and places led to almost identical results.

Effect of Propylthiouracil and of Desiccated Thyroid on the Development of Liver Necrosis.—The role of hyperthyroidism in the causation of liver damage has been a controversial subject for many years. In 1921, Hashimoto [37] reported the production of centrolobular necrosis in rats that received 100 to 500 mg. of thyroid tissue per day. On the other hand, Davis and Whipple [38] found that excessive

32. Lindan, O., and Work, E.: Biochem. J. **48**:337 and 344, 1951.

33. Ness, H. T.; Price, E. L., and Parsons, N. T.: Science **103**:198, 1946.

34. Type 50 B brewer's yeast, Standard Brands, Inc., New York.

35. D. C. L. Vitamin B_1 yeast, Distillers Company, Ltd., Glasgow, Scotland; or Type 753-powdered Fleischmann's baker's yeast, Standard Brands, Inc., New York.

36. U. S. P.-grade brewer's yeast, Nutritional Biochemicals Corporation, Cleveland.

37. Hashimoto, H.: Endocrinology **5**:579, 1921.

38. Davis, N. C., and Whipple, G. H.: Arch. Int. Med. **23**:612 (May) 1919.

amounts of thyroid did not potentiate the action of hepatoxic agents, such as chloroform, on the livers of dogs. This finding was later confirmed in rats by Cameron and Karunaratne.[39] Later, however, McIver [40] concluded that rats maintained on a standard diet and treated by injection of crystalline thyroxine or fed desiccated thyroid tissue showed an increased susceptibility to chloroform poisoning. In contrast to the conflicting reports in the experimental field there would appear to be less doubt with regard to the relationship between hyperthyroidism and liver dysfunction in the clinical field.[41]

Recently, the effects of thyroidectomy and of thiouracil on liver lipids [42] and of thiouracil and propylthiouracil on the development of liver cirrhosis in rats [43] have been reported by Handler and by György and his associates respectively. The former investigator, in collaboration with Follis,[44] has tested also the effect of desiccated thyroid on the development of liver necrosis and has concluded that "thyroid feeding hastened the death of cystine-deficient rats, and their livers exhibited the acute necrosis characteristic of this deficiency." The following experiment gives further information on the effects of increasing and decreasing the metabolic rate in rats on necrogenic diets by supplementing the diets with desiccated thyroid and propylthiouracil respectively.

The data in Table 3 show that the inclusion of desiccated thyroid (0.3%) in the diet potentiated greatly the necrogenic property of the basal diet by causing death due to necrosis at an average of only 20 days, compared with 39 days for the basal diet (Groups DT and W). This finding is in agreement with the work of Handler and Follis.[44] Presumably the potentiating action of desiccated thyroid may be explained on the basis of increased metabolic activity and consequently an increased demand for essential protective nutrients.

The results of supplementing with propylthiouracil (0.3%), on the other hand, are somewhat difficult to interpret. None of the animals died of liver necrosis, but they all died of undetermined causes at an average of 47 days, an interval much longer than that required for liver damage to develop in the animals on the basal diet. If one assumes that this is a real antinecrogenic effect, the latter may be explained either by its antithyroid action, or, since it has been shown by György and associates to exist in tautomeric form,[45] by its ability to supply sulfhydryl groups to the animal body. Handler and Follis [44] found that a level of 0.25% thiouracil permitted the animals to survive the experimental period of 120 days and prevented the development of hepatic necrosis. The effects of desiccated thyroid and of propylthiouracil in the development of liver necrosis are being further investigated.

Effect of Sulfaguanidine and Aureomycin on the Development of Liver Necrosis.—The greatest degree of tissue destruction is usually in the left half of the liver, and this section receives its portal blood flow from the left half of the large intes-

39. Cameron, G. R., and Karunaratne, W. A. E.: J. Path. & Bact. **41**:267, 1935.
40. McIver, M. A.: Proc. Soc. Exper. Biol. & Med. **45**:201, 1940.
41. Lichtman, S. S.: Ann. Int. Med. **14**:1199, 1941.
42. Handler, P.: J. Biol. Chem. **173**:295, 1948.
43. György, P., and Goldblatt, H.: Science **102**:451, 1945. György, P.; Rose, C. S., and Goldblatt, H.: Proc. Soc. Exper. Biol. & Med. **67**:67, 1948.
44. Handler, P., and Follis, R. H., Jr.: J. Nutrition **35**:669, 1948.
45. György, P.; Stiller, E. T., and Williamson, M. B.: Science **98**:518, 1943.

tine, the spleen, and the stomach.[46] This circumstance led Dent [47] to postulate that some endotoxin might be given off from the intestine drained by this part of the portal system. The administration of sulfaguanidine [48] or aureomycin [49] is well known to lower markedly the bacterial count in the intestinal tract and hence, if the intestinal flora was responsible for the production of such a hepatotoxin, these substances should exert a prophylactic action in dietetic hepatic necrosis.

Table 3 shows the results of including a supplement of 1% sulfaguanidine (Group SG). Of the 10 animals that received the test diet, 6 died from other causes prior to the number of days at which death from liver necrosis occurred in the group; 3 died of liver necrosis at an average interval of 96 days, compared with 39 days on the basal diet, and 1 survived the experimental period with no signs of liver necrosis. There would appear to be little doubt, therefore, that sulfaguanidine exerted a definite ameliorative effect on the necrogenicity of the basal diet. The results of supplementing the basal diet with 0.625% aureomycin gave qualitatively similar results (Table 4, Groups AM and W). The incidence of necrosis was essentially unchanged, but the average time required for the occurrence of death due to liver damage was definitely longer for the group whose diet was supplemented (67 days vs. 41 days).

TABLE 4.—*Effect of Aureomycin and Vitamin B_{12} on the Development of Liver Necrosis*

Group	Rats	Av. Initial Wt., Gm.	Dietary Supplement	Rats That Died of Necrosis or Were Killed When Moribund		Rats Killed at 104-106 Days		Total Incidence of Necrosis (Corrected) *	
				Rats	Days on Diet (Av. Range)	No. Showing Signs of Necrosis	No. Showing No Signs of Necrosis	No. of Rats	Per Cent
W	5	63	None	5	41 (37-44)	..	..	5/ 5	100
AM	10	63	0.625% aureomycin	9	67 (49-78)	..	1	9/10	90
BO	10	64	B_{12}—1γ/rat/day in diet	8	55 (40-85)	..	..	8/ 8	100 †
BI	10	63	B_{12}—1γ/rat/day by injection	8	49 (44-59)	..	..	8/ 8	100 †

* See footnote to Table 1.
† Four animals died of intercurrent infections and showed no evidence of dietary necrosis. Two were from Group BO and died on the 30th and 36th days; two were from Group BI and died on the 8th and 25th days.

These results could be interpreted as supporting the possibility that a bacterial toxin was responsible for the production of dietetic liver necrosis. On the other hand, MacKenzie and associates [50] demonstrated that sulfaguanidine causes thyroid hyperplasia and a decrease in the metabolic rate of rats. In the light of the findings reported in this paper on the effect of desiccated thyroid and propylthiouracil on the development of liver necrosis it is possible that the action of sulfaguanidine in ameliorating the necrogenic quality of the basal diet was brought about by its effect on the thyroid gland. Information concerning the effect of aureomycin in

46. Copher, G. H., and Dick, B. M.: Arch. Surg. **17**:408 (Sept.) 1928. Glynn, L. E., and Himsworth, H. P.: J. Path. & Bact. **56**:297, 1944.

47. Dent, C. E.: Biochem. J. **41**:314, 1947.

48. Marshall, E. K.; Bratton, A. C.; White, H. J., and Litchfield, J. J.: Bull. Johns Hopkins Hospital **67**:163, 1940.

49. Sborov, U. M.; Joy, A. R., and Watson, C. J.: Proc. Central Soc. Clin. Research **22**:70, 1949.

50. Mackenzie, J. B.; Mackenzie, C. G., and McCollum, E. V.: Science **94**:518, 1941. Mackenzie, C. G., and Mackenzie, J. B.: Endocrinology **32**:185, 1943.

this regard appears to be lacking. The finding that the inclusion of aureomycin in a basal necrogenic diet lessens its necrogenicity confirms the recent report by György and associates [51] on this point.

Effect of Vitamin Supplements on the Development of Liver Necrosis.—The possibility that the presence or the absence of certain vitamins might play a role in the development of liver necrosis seemed worthy of investigation. The vitamin supplement of the basal diet, which contained thiamine hydrochloride, riboflavin, pyridoxine hydrochloride, and calcium pantothenate, was modified by the addition of nicotinic acid, paraaminobenzoic acid, folic acid, biotin, and vitamin B_{12} in the amounts indicated in the fourth footnote to Table 3. It was realized, of course, that most of these substances were present in significant amounts in the yeast component of the basal diet, but apart from the question of physiological availability of the vitamins in this component of the diet, we were also concerned with working toward an adequate synthetic diet for the problem under investigation. An examination of Table 3 shows that the group whose diet was thus supplemented did not differ from that fed the basal diet (Groups VM and W respectively).

A prominent histological feature observed in the livers of animals fed the basal necrogenic diet and killed prior to the development of necrosis was a decrease in the number and staining intensity of the basophilic granules found in normal liver cells.[9] Stern, Taylor, and Russell [52] reported that vitamin B_{12} prevented the loss of the basophilic granules from the liver cells of rats fed diets low in protein. These observations and the report by Popper, Koch-Weser, and Szanto [53] that vitamin B_{12} protected rats against fatty metamorphosis and liver damage associated with carbon tetrachloride poisoning prompted the testing of the effect of the addition of this vitamin, in relatively large amounts, on the necrogenic property of the basal diet. The vitamin was given at a level of 1 γ per rat per day in the diet and by injection to Groups BO and BI respectively (Table 4). Although neither method of B_{12} supplementation brought about any change in the incidence of necrosis, the average period before death due to liver necrosis occurred was longer than for the group whose diet was unsupplemented—55, 49, and 41 days for Groups BO, BI, and W respectively (Table 4). These differences could not be shown to be significantly different. In a similar experiment, György and Rose [54] found that the incidence was unchanged by the inclusion of B_{12} and that there was no difference in the time required for death to occur because of hepatic damage—36 days for the group that received B_{12} versus 34 days for the basal group.

SUMMARY

In an attempt to elucidate further the factors involved in the production of massive liver necrosis in rats fed diets low in methionine, cystine and alpha-tocopherol, experimental conditions have been varied with regard to initial weight and sex of the test animal and with regard to various dietary supplements.

51. György, P.; Stokes, J.; Smith, W. H., and Goldblatt, H.: Am. J. M. Sc. **220**:6, 1950.

52. Stern, J. R.; Taylor, M. W., and Russell, W. C.: Proc. Soc. Exper. Biol. & Med. **70**:551, 1949.

53. Popper, H.; Koch-Weser, D., and Szanto, P. B.: Proc. Soc. Exper. Biol. & Med. **71**:688, 1949.

54. György, P., and Rose, C. S.: Proc. Soc. Exper. Biol. & Med. **73**:372, 1950.

The greater the initial weight of the rat, the longer the time required for liver necrosis to develop. Female rats were found to be definitely more resistant to hepatic damage than males.

The inclusion of cod liver oil greatly increased the necrogenic quality of the basal diet.

Of the lipotropic and antilipotropic substances tested, choline, inositol, cholesterol, and cystine, only the last had any effect on the development of liver necrosis. This substance displayed its well-known prophylactic action.

In confirmation of previous reports, alpha-tocopherol, like cystine, prevented the development of hepatic damage. Alpha-tocopherol quinone did not act as a structural antagonist to alpha-tocopherol in the development of liver necrosis, and indeed it actually caused a diminution in the necrogenicity of the basal diet. A similar but definite effect was noted for synthetic vitamin K (menadione).

None of the purines, uric acid, theophylline, or xanthine had any prophylactic action.

A tyrosine supplement (1%) had no demonstrable effect, but the addition of ascorbic acid (0.625%) decreased slightly the necrogenicity of the basal diet.

Heat inactivation of active baker's yeast did not alter the effect of the basal diet but substitution of brewer's yeast for the baker's yeast caused a decrease in its necrogenicity.

Supplementation with desiccated thyroid (0.3%) greatly increased the necrogenic property of the basal diet, causing death from hepatic necrosis in half the normal time. The antithyroid drug, propylthiouracil, on the other hand, appeared to prevent liver necrosis, although the animals died of other causes after an interval on the test diet greater than that required to induce necrosis in rats on the basal diet.

Sulfaguanidine and aureomycin both caused definite amelioration of the necrogenic property of the basal diet.

With the exception of vitamin B_{12} alterations in the vitamin supplement had no effect. In the case of B_{12} there appeared to be a slight increase in the time required for death due to liver necrosis, but this difference could not be shown to be of statistical significance. The implications of some of these results are discussed.

DYNAMICS OF INFLAMMATION AND OF REPAIR

II. Chemotactic Substances in Normal Tissues

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MEDICAL literature contains numerous observations on chemotaxis. The fact that various pathogenic bacteria attract leucocytes naturally focused attention on chemotaxis as a phenomenon of inflammation of infectious origin. For this reason investigators were most interested in the chemotactic effects of various bacteria. (See Wells [1] and McCutcheon [2] for reviews.) Studies were made for chemotactic effects, not only on bacteria and their products but on protozoa and yeasts, also on histamine, starch and other carbohydrates, polysaccharides, agar, cotton, silk, collodion, oils, stearic acids, inorganic chemicals such as silica, kaolin, carbon, titanium dioxide, manganese dioxide, and others. It seems remarkable that these investigations did not include animal cells and tissue substances among those tested.

It is well known that diverse forms of uninfected injuries—mechanical, chemical, thermal, electrical, and others—result in inflammation accompanied by leucocytic infiltration. Few reports have been published concerning the chemotactic effects of substances derived from normal or injured cells. Moon [3] produced sterile blebs on human skin by burning and noted that leucocytes promptly migrated into the blister fluid. Similar burns on animal skin, under conditions of surgical asepsis, resulted in rapid leucocytic infiltration of the lesions. Since no bacteria or foreign matter of any kind had been introduced, he concluded that substances derived from injured cells had attracted the leucocytes into the area. Grand and Chambers [4] noted that leucocytes were attracted toward and into microcapillary tubes filled with an extract of muscle in Tyrode's solution. Muscle fibers in tissue culture, if mechanically injured, attracted leucocytes. They concluded that injured tissues

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2. McCutcheon, M.: Chemotaxis in Leukocytes, Physiol. Rev. **26**:319, 1946.
3. Moon, V. H.: Mechanism of Acute Inflammation, Arch. Path. **20**:561 (Oct.) 1935.
4. Grand, C. G., and Chambers, R.: Chemotactic Reactions of Leukocytes to Irritated Tissues, J. Cell. & Compt. Physiol. **9**:165, 1937.

liberate substances which attract polymorphonuclear leucocytes. Silverman [5] investigated normal human skin by the method devised by McCutcheon and associates.[6] He found that normal dermal cells contained a water-soluble substance which had a chemotactic index equal to that of pathogenic bacteria. Menkin [7] produced sterile purulent exudates in dogs and, by chemical cleavage, derived from these a thermostable, dialyzable substance which attracted leucocytes both in vivo and in vitro.

These observations indicate that various tissues and cells contain substances which attract leucocytes. Since this field has not been explored adequately, it seemed desirable to investigate the chemotactic properties of products derived from normal tissues.

Using the cover-slip method devised by McCutcheon and associates, we found that various uninfected normal tissues attract leucocytes. Since this technique does not readily provide quantitative data, we made tentative experiments with capillary tubes. These had been used in varying techniques by earlier workers (Häbler [8]; Massart [9]; Bloch [10]; Schade and Mayr [11]; Meier [12]), but results were regarded as unreliable by some. We tried various modifications in an effort to eliminate sources of error in previous methods and to provide adequate controls on the results. The following technique was evolved by repeated trials and modifications.

METHODS

Various organs and tissues were obtained from a freshly killed normal rabbit. The tissue was ground through a meat chopper or was pulped with mortar and pestle, then suspended in 4 volumes of isotonic sodium chloride solution and beaten to a fine suspension in an electric disintegrator (Waring blendor®). The suspension was then centrifuged, and the supernatant fluid was adjusted to pH 7 and sterilized by filtration (Seitz). This watery extract was kept under refrigeration at 3 to 4 C. until used.

Blood was drawn by cardiac puncture from a normal rabbit into an anticoagulant and was centrifuged immediately at moderate speed. This gave a supply of fresh rabbit plasma and of leucocytes from the buffy coat overlying the red corpuscles. Part of the plasma was used in suspending the leucocytes, and part was reserved for another use. In other instances, leucocytes were obtained by injecting saline solution into the abdomen of a rabbit and withdrawing the fluid a few hours later.

One end of a capillary coagulation tube (A. H. Thomas No. 3467, outside diameter 1.0 to 1.25 mm.) was drawn out in a microflame to a fine capillary tip. The total length of this tube was about 65 mm., including about 5 mm. of tapered tip.

In making a test the large end of the capillary tube was dipped into the tissue extract until, by capillary attraction, the tube was filled to a distance of about 30 mm. It was then dipped into normal rabbit plasma until it was filled almost to the end of the capillary tip. This tip was then sealed off in a microflame, while the opposite end of the tube was immersed in plasma.

5. Silverman, D.: A Chemotropic Substance Derived from Normal Tissues, Arch. Path. **25**:40 (Jan.) 1938.

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When the sealed tip was thoroughly cooled, the open end was inserted deeply into a suspension of fresh leucocytes in a test tube and then incubated at 38 C. in a water bath for three hours. The water bath was kept covered in order to minimize any difference in temperature between the water and the air surrounding the capillary tubes. The test tubes were shaken gently at intervals to counteract the settling of the leucocytes by gravity. Controls were prepared by filling capillary tubes entirely with plasma; these were then sealed and treated as just described.

After incubation the end of the capillary tip was snipped off and the entire contents of the tube were extruded onto a hollow-ground slide and drawn into a hemocytometer pipet for counting white corpuscles. The content of the pipet then was diluted as in making leucocytic counts on blood, and the number of leucocytes per cubic millimeter was determined in the counting chamber.

The conditions of these experiments provided a column of plasma about 30 mm. in height between the leucocytes and the extracts to be tested. Since leucocytes have a higher specific gravity than either extracts or plasma, any effective chemotaxis must require them to rise in the tube against the force of gravity. It seems unlikely that floating of leucocytes was a factor in the results; nor do convection currents seem probable factors. The effects of these or of other disturbing influences would appear in the controls.

It soon was found that varying numbers of lymphocytes wandered into the plasma in the control tubes. Others have found that lymphocytes are highly motile and are not influenced by chemotactic attraction. This disturbing factor was minimized by absorbing on filter paper about 5 mm. of length of fluid column from the lower end of the tube, and making counts of leucocytes in the remainder. This precaution was applied to all tubes, both test and control.

Some minor details facilitated the technical procedures described. In order to hold the open ends of the capillary tubes at an equal depth in the tube containing leucocytes, four or five perforations were made with a coarse needle in a bit of cardboard about 2.5 cm. square. The capillary tubes were then inserted through the perforations, and the cardboard was laid across the top of the tube containing the leucocytic suspension. By this device, all tubes were held immersed at the same level in the leucocytic suspension.

To facilitate extruding the fluid from the capillary tubes, the following method was devised: The rubber tube of a hemocytometer pipet was slipped over the tip of a medicine dropper, the rubber bulb of which had been punctured with a coarse needle. After snipping the sealed tip of the capillary pipet, the latter was inserted through the puncture in the bulb. By blowing gently through the mouthpiece, the fluid in the capillary pipet was extruded into the hollow-ground slide, whence it was drawn up readily into a hemocytometer pipet. Regularly there was sufficient fluid to fill the hemocytometer pipet to the 0.5 mark.

In the earlier part of these experiments the tissue extracts had been sterilized by filtration but otherwise no aseptic precautions were taken. Later, aseptic precautions were applied throughout. All the solutions, syringes, needles, capillary tubes, and glassware were sterilized by autoclave, by boiling, or by dry heat. The results given below are those obtained under conditions of bacteriologic asepsis.

RESULTS

It soon was evident that, while each of the tissues tested showed chemotactic properties, the degree of attraction for leucocytes varied greatly. Table 1 is a condensation of the results obtained under aseptic technique.

It was found that the activity of leucocytic suspensions varied somewhat from day to day. In order to distribute this variation, extracts of several tissues, together with controls, were set up in a series each day. A usual setup was as follows: four capillary tubes each for three tissues, as lung, liver, and brain, and four controls.

Regularly there were moderate variations in the leucocytic counts both in the tests and in the controls. The latter often resulted: 50, 0, 50, 100. These seemed higher than one would expect until one recalls that both neutrophils and lymphocytes are actively motile and do much random wandering. We have verified the observation of McCutcheon and associates that these cells move about at random, especially when no chemotactic substance is present. Accordingly, it may be

expected that leucocytes in moderate numbers will wander into the control tubes which contain only plasma. If one assumes that random wandering is equal in both tests and control tubes, one may subtract the number for the controls from that of the tests. This leaves a high count of leucocytes whose presence indicates some influence not supplied in the controls.

Instituting aseptic technique made only slight differences in the leukocytic counts. The average count in eight aseptic tests on spleen was 581.5 against an average count of 600.0 in eight tests without asepsis. The average count in 20 aseptic tests on intestinal mucosa was 351.0, compared with 352.7 in 10 tests without asepsis. The average count in 88 control tubes under aseptic conditions was 78.7, compared with 89.2 in 64 controls before aseptic technique was begun.

TABLE 1.—*Degrees of Chemotaxis Shown by Tissue Extracts Tested with Leucocytic Suspensions*

Extract	Tests	Av. Count per Cubic Millimeter
Lung	89	683.2
Skin	25	561.6
Kidney	24	499.2
Spleen	24	515.4
Gastric mucosa	20	450.0
Intestinal mucosa	20	351.0
Brain	16	313.6
Muscle	24	295.6
Adrenal	8	250.0
Liver	20	208.4
Testes	24	201.0
Controls	88	78.7

TABLE 2.—*Effect of Dilution of the Tissue Extract on Chemotaxis*

	Av. Count with Stated Dilution			
Extract	Undiluted	1:5	1:25	1:125
Lung	550.0	325.0	75.0	50.0
Skin	425.0	200.0	50.0	50.0
Controls	50.0	.....	....	....
Kidney	575.0	100.0	00.0	25.0
Spleen	375.0	175.0	25.0	25.0
Controls	75.0	.....	....	....

It was found that the degree of chemotaxis shown was not proportional to the cell count on the leucocytic suspension. For example, four tests on extract of lung with a leucocytic suspension counting 25,300 cells per cubic millimeter gave an average count of 562.5, whereas four tests of the same extract with the same leucocytes diluted with plasma to a count of 9,950 cells per cubic millimeter gave an average count of 475.0. This figure should have been 225 if the degree of chemotaxis were proportional to the cell count in the leucocytic suspension. Again: two tests on extract of intestinal mucosa counted 250 and 350 cells, respectively, with a leucocytic suspension counting 6,600 cells per cubic millimeter. Two tests of the same extract with a suspension counting 19,950 cells per cubic millimeter showed 250 and 300 cells, respectively. Two tubes of lung extract gave figures of 500 and 600 cells with a leucocytic suspension of 11,500 cells per cubic millimeter and figures of 700 and 800 with a suspension of 6,450 cells per cubic millimeter.

While chemotactic activity is not directly proportional to the concentration of leucocytes in the suspension, the same does not apply to the concentration of the tissue extract. Table 2 lists the results obtained when various dilutions of the extract were tested simultaneously with the same leucocytic suspension.

Extracts of some tissues showed a moderate loss of chemotactic activity after storage at 3 to 4 C. For example, extract of lung decreased from 981.2 to 525.0, average of four tests, in eight weeks. Extract of skin decreased from 650.0 to 525.0 in eight weeks. Extracts of other tissues lost only slightly, and some were as active after as before eight weeks' refrigeration.

Extracts varied widely in their susceptibility to heat. The differences are shown in Table 3.

TABLE 3.—*Effect of Heat on the Chemotaxis of Tissue Extracts*

Extract	65 C., 30 Min.	80 C., 30 Min.	100 C., 10 Min.
Lung	No decrease	Slight decrease	No chemotaxis
Kidney	No decrease	No chemotaxis	.....
Spleen	No decrease	Marked decrease	No chemotaxis
Muscle	Slight decrease	No chemotaxis	.....
Skin	No decrease	Marked decrease	No chemotaxis
Brain	No chemotaxis	.....	.....
Adrenal	No chemotaxis	.....	.....
Testes	No chemotaxis	.....	.....
Liver	No chemotaxis	.....	.....
Intestinal mucosa	No chemotaxis	.....	.....

TABLE 4.—*Influence of Species Variation on Chemotaxis of Tissue Extracts Tested with Leucocytic Suspensions*

Extract	Leukocytes	Av. Count
Human skin	Human	457.5
Human skin	Rabbit	225.0
Rabbit skin	Human	187.5
Rabbit skin	Rabbit	561.8
Rabbit lung	Human	300.0
Rabbit lung	Rabbit	683.2
Rabbit spleen	Human	200.0
Rabbit spleen	Rabbit	515.4
Rabbit kidney	Human	237.5
Rabbit kidney	Rabbit	499.2

It is seen from these data that the chemotactic substance of some tissues is inactivated by heating to 65 C. for 30 minutes while that in other extracts was not affected by heating to 65 but required 80 or 100 C. to inactivate. These temperatures caused precipitation of the protein present in the extract; the tests were made on the fluid from which the precipitate had been filtered out. Hence it does not follow that the chemotactic substance was destroyed; it may only have been precipitated. But the fluid from lung, kidney, muscle, and skin was chemotactic after heating to 65 C. and lung, spleen, and skin were not entirely inactivated at 80 C. Hence it is concluded that the chemotactic substance is not identical in all tissues and that it is thermolabile to varying degrees. In this particular the chemotactic substance is not identical with "leukotaxin," which has been described as thermostabile.[7]

One series of tests indicated that chemotaxis of tissue substances depends somewhat on species. Tests were made using human leucocytes and plasma against extracts of rabbit tissues, and vice versa. The results are compared in Table 4. The data indicate that when leucocytes and tissue extracts are heterologous, chemotaxis is decreased about 50% below the figures resulting when homologous materials are used.

COMMENT

The data resulting from our experiments have raised several questions, the answers to which are not apparent. Why should the chemotactic activity of different tissues vary so widely? The data are not based on one batch of extract but on several batches from each tissue. Hence the high average counts obtained with lung, skin, kidney, spleen, and gastric mucosa as compared with other tissues are, we believe, due to some specific but unknown difference in the tissues themselves. One might suppose that tissues such as skin, lung, and gastric mucosa are more subject than others to various extrinsic injuries; hence they had more frequent need to issue an alarm which would activate defensive forces. But this suggestion would not apply to the spleen and the kidney, extracts of which are also highly chemotactic.

Why should extracts of liver and of testes give results only moderately higher than the controls? The answers are not apparent, one can only record what resulted under the conditions of the experiments.

It has been observed that leucocytic infiltration is not a conspicuous feature of uninfected hepatic necrosis such as that seen in passive congestion, poisoning with carbon tetrachloride, chloroform, phosphorus, "acute yellow atrophy," or viral hepatitis. Could it be that low chemotactic activity of liver substance may account for the relative absence of leucocytic infiltration in these conditions? An abundance of leucocytes is found in hepatic abscesses due to pyogenic infections, but here the organisms may supply the chemotactic attraction. The answers to these questions must await further evidence.

SUMMARY AND CONCLUSIONS

A technique not used hitherto for chemotactic experiments is described. It has the advantage of providing numerical results and controls.

Normal animal tissues contain water-soluble substances which attract neutrophils in vitro. The amount or the activity of these substances varies widely in different tissues.

The numerical values obtained are not directly proportional to the density of the leucocytic suspensions used.

The tissue extracts used were inactivated by heat at different degrees. This indicates that the chemotactic substances in different tissues are not identical.

Moderate loss of chemotactic activity results in varying degrees from prolonged storage in refrigeration.

Chemotactic activity is more pronounced when homologous materials are used. It is less pronounced when the leucocytes and the tissue extracts are obtained from different animal species.

The fact that normal tissues contain chemotactic substances supports the explanation that cellular substances released from damaged tissues act as the trigger mechanism which initiates acute inflammation.

EFFECT OF VARIOUS PATHOLOGICAL CONDITIONS ON THE COPPER CONTENT OF HUMAN TISSUES

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THE SIGNIFICANCE of the presence of copper in the tissues of the higher forms of life is but incompletely understood. According to Stotz, Harrer, and King,[1] it is the active substance in the so-called "ascorbic acid oxidase." Oxyhemocyanine, the blue oxygen-carrying pigment of the blood of snails and crabs, contains copper rather than iron. In the course of evolution, the significance of copper for hemopoiesis has not been entirely lost, for iron must be supplemented with copper in order that rats may recover from the nutritional anemia which is produced by a diet deficient in copper.[2]

Despite some early conflicting reports, the findings of Hart, Steenbock, Waddell, and Elvehjem [2] concerning the importance of copper in hemoglobin regeneration have been repeatedly confirmed in many other laboratories. Keil and Nelson [3] by the administration of copper alone to anemic rats demonstrated a lowering of the peak of their glucose tolerance curves, indicating an acceleration of glycogen formation and thus a more rapid removal of glucose from the blood.

It has been reported that copper interferes with the action of several enzymes, including lipase,[4] intracellular diastase,[5] and certain of those connected with the metabolism of glucose.[6]

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Sachs and associates [7] have reported a relationship between copper and iron levels in the blood of humans. They observed that when the iron content decreases, the copper content increases. This was shown to hold true in a large series which included cases of pernicious anemia, sickle cell anemia, and leukemia. They also observed hypercupremia in pregnancy and called attention to the fact that anemia often accompanies pregnancy. The relationship of copper to hemochromatosis has been the subject of much controversy. Mallory and Parker [8] claimed that hemochromatosis was a result of chronic copper poisoning, and in support of this contention they reported that the hepatic pigment deposits in rats, which had received food rich in copper, simulated those deposits found in man having hemochromatosis. The findings of Hall and Butt [9] were in substantial agreement with those of Mallory; however, neither of these investigators employed normal controls.

Flinn and Von Glahn [10] and Polson,[11] who repeated the experiments of Mallory and Parker, were unable to confirm findings of the latter investigators and concluded that copper was not the cause of hemochromatosis. These workers (Flinn and associates) observed that the administration of sodium acetate without copper produced the same type of lesions as followed the administration of copper acetate.

Sheldon,[12] who studied 311 cases of hemochromatosis, concluded that the copper content in alcoholic beverages was not the causative agent for hemochromatosis as Mallory and Parker had suggested. Funk and St. Clair [13] reported finding 14 mg. of copper per 100 gm. of fresh tissue in a case of hemochromatosis. This was the only quantitative determination of copper reported by the various investigators in studies of hemochromatosis.

A review of the literature has shown a paucity of information relative to the copper content in human tissues. Flinn and Von Glahn [10] reported the content of copper in 20 human livers as varying from 0.23 to 1.24 mg. per 100 gm. of fresh tissue. Hahn and Fairman [14] analyzed 13 human livers for copper by the chromotropic acid method as described by Ansbacher, Remington, and Culp [15] and found that the copper content varied from 0.33 to 7.8 mg. per 100 gm. of fresh tissue. Brüchmann and Zondek [16] reported 3.5 mg. of copper per 100 gm. of

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fresh hepatic tissue in a case of diabetes mellitus. Gerlach [17] concluded that the copper content of the liver was not significantly changed in hepatic cirrhosis, tuberculosis, or blood dyscrasias.

From the above limited and available data it appears that there is little agreement as to what levels of copper constitute the normal limits in human tissues. It is important from a toxicological point of view to know what the normal levels are so as to know what constitute abnormal concentrations. This study is primarily concerned with the establishment of normal copper levels in the liver and the kidney, since these organs are those usually employed for toxicological examinations.

METHODS AND MATERIALS

In all cases the tissues were obtained directly in the autopsy room, in order to avoid any possible contamination, and were immediately placed in a refrigerator. They were analyzed within a period of 72 hours after being removed from the body. In all the necropsies revealing neoplasms, the specimens for analysis were taken not only from the tumor areas but from areas showing no gross evidence of disease. Approximately 3 to 5 gm. of tissue were placed in 300-ml. Kjeldahl flasks and digested with 2 ml. of metal-free concentrated sulfuric acid with repeated additions of metal-free concentrated nitric acid until the organic matter was completely destroyed. About 50 ml. of metal-free water was then added to the flasks and the mixture boiled for several minutes until the precipitated salts were dissolved. The specimens were transferred to volumetric flasks and aliquots saturated with sulfur dioxide to reduce the iron, which was the principal interfering element. The solutions were adjusted approximately to pH 8.4 with metal-free ammonium hydroxide, cresol red being used as the indicator. The copper was extracted with 25 ml. of purified dithizone dissolved in carbon tetrachloride and the extract transferred to a separatory funnel. The excess dithizone was removed by washing with approximately 50 ml. of metal-free water made slightly alkaline with several drops of metal-free ammonium hydroxide. The copper dithizonate in carbon tetrachloride was then filtered through a dry copper-free No. 40 Whatman filter paper into a colorimeter tube and readings taken on an Evelyn colorimeter employing a 5,200 A. filter. Duplicate readings did not vary more than + or — 2.5%.

RESULTS

Table 1 shows that there were 46 necropsies in which neither renal nor hepatic pathological changes were noted and that in 12 of the subjects, varying in age from stillborn to 17 years, the hepatic copper level varied from 11.70 to 26.75 mg. and the renal level from 12.50 to 30.82 mg. per 100 gm. of tissue. For toxicological purposes these levels might be considered within normal limits. In one other of this age group, with a diagnosis of interstitial pneumonia (Necropsy 49:268), the liver contained 4.52 mg. and the kidney 6.29 mg. of copper per 100 gm. of fresh tissue. These levels undoubtedly are below average. In 33 subjects, varying in age from 17 to 80 years, the copper content of the liver ranged from 10.88 to 28.80 while in the kidney it varied from 17.75 to 30.76 mg. per 100 gm. of fresh tissue. In one other of this age group (Necropsy 50:224), whose condition was diagnosed as a reticulum cell sarcoma, the liver contained only 3.5 mg. of copper, while the kidney contained 12.50 mg. of copper, per 100 gm. of fresh tissue.

In the 35 necropsies listed in Table 2, showing evidence of renal pathological changes but no pathological alterations of the liver, the copper content of the

17. Gerlach, W.: Untersuchungen über den Kupfergehalt menschlicher (und tierischer) Organe, Arch. path. Anat. **294**:171, 1935.

kidneys was found to be in the range of 10.51 to 28.75 mg. per 100 gm. of tissue except in Necropsy 49:242, in which the kidneys were found to contain 5.85 mg. per 100 gm. of fresh tissue. The hepatic copper levels were all in the range of

TABLE 1.—*Concentrations of Copper in Liver and Kidney in the Absence of Morphological Abnormalities in These Organs*

Autopsy	Age	Sex	Kidney *	Liver *	Anatomic Diagnosis
50:112	Stillborn	F	18.50	18.75	Erythroblastosis fetalis
50:118	Stillborn	F	13.50	16.50	Stillbirth
49:253	9 hr.	M	13.50	18.92	Multiple skull fractures
49:206	3 days	M	12.80	13.70	Atresia of esophagus
50:209	3 days	F	18.50	15.00	Erythroblastosis fetalis
49:265	30 days	M	13.80	18.70	Ichthyosis
50:210	3 mo.	F	14.29	13.50	Intestinal adhesions
49:268	7 mo.	F	6.29	4.52	Interstitial pneumonia
50:58	7 mo.	M	14.75	13.65	Congenital heart disease
50:111	7	F	20.50	18.70	Benign cyst of pituitary
50:72	11	M	30.82	26.75	Coaretation of aorta
50:212	15	M	18.75	12.25	Astrocytoma
50:218	16	M	12.50	11.70	Spongioblastoma
49:216	63	M	28.17	26.69	Glioblastoma
49:222	30	F	26.70	24.95	Thrombocytopenic purpura
49:225	78	M	25.12	23.30	Generalized arteriosclerosis
49:237	27	M	26.70	28.75	Laparotomy wound open
49:256	78	M	20.32	20.58	Generalized arteriosclerosis
49:263	60	F	20.80	21.20	Coronary arteriosclerosis
50:11	23	M	27.30	23.70	Chronic colitis
50:12	56	M	25.20	23.40	Pericarditis
50:33	17	F	21.29	17.20	Congenital cardiac anomaly
50:49	25	M	28.75	23.45	Congenital aneurysm of cerebral artery
50:51	55	F	27.60	28.80	Adenocarcinoma of stomach
50:53	65	M	24.12	27.50	Thrombosis of pulmonary and hepatic veins
50:57	63	M	28.75	22.81	Thrombosis of inferior vena cava
50:65	52	M	28.75	23.80	Coronary arteriosclerosis
50:68	60	M	30.76	26.28	Carcinoma of stomach
50:70	63	F	29.75	28.62	Lymphoma of spleen and liver
50:76	76	M	18.25	19.75	Carcinoma of prostate
50:77	45	M	17.85	20.50	Duodenal ulcer
50:89	57	M	22.67	15.08	Hodgkin's disease
50:92	60	M	17.75	10.88	Leukemia
50:106	40	M	18.09	26.50	Astrocytoma of cerebellum
50:91	38	M	18.75	16.50	Lymphoma of spleen and lymph nodes
50:90	51	M	30.51	27.75	Prefrontal lobotomy
50:108	53	M	18.50	21.50	Generalized arteriosclerosis
50:113	55	M	18.50	22.25	Neoplasm of frontal lobe
50:115	52	M	19.50	18.70	Glioblastoma multiforme
50:116	80	M	18.70	20.15	Myocardial infarction
50:123	60	M	18.70	21.89	Carcinoma of head of pancreas
50:125	35	M	20.50	18.65	Acute leukemia
50:204	64	M	22.50	18.70	Perforated gastric ulcer
50:206	45	F	18.70	19.25	Glioblastoma multiforme
50:208	31	M	18.09	20.15	Acute poliomyelitis
50:224	62	M	12.50	3.50	Reticulum cell sarcoma

* The concentrations of copper are expressed in milligrams per 100 gm. of fresh tissue.

12.51 to 31.79 per 100 gm. tissue except in two subjects having renal neoplasms and in two who were diabetic. In the two cases of renal neoplasm, the liver contained 5.15 and 5.85 mg. of copper per 100 gm. of fresh tissue, while in the cases of diabetes the liver contained 1.75 and 3.20 mg. of copper per 100 gm. of fresh

tissue. The hepatic and renal copper levels were unusually high in a case of renal adenomyosarcoma—63.62 mg. per 100 gm. of liver and 73.70 mg. per 100 gm. of kidney.

TABLE 2.—*Concentrations of Copper in Liver and Kidney When There Was Morphological Evidence of Abnormality in the Kidney While the Liver was Essentially Normal*

Autopsy	Age	Sex	Kidney*	Liver*	Morphologic Changes in Kidney	Anatomic Diagnosis
49:212	33	M	16.27	20.65	Chronic pyelonephritis	Bronchopneumonia
49:264	67	M	11.79	31.79	Pyelonephritis	Pulmonary thrombosis
50:86	77	M	23.68	20.50	Chronic pyelonephritis and hydronephrosis	Adenocarcinoma of prostate
50:83	74	M	14.50	17.50	Chronic pyelonephritis	Basophilic adenocarcinoma of pituitary
50:88	23	M	22.87	18.85	Chronic pyelonephritis	Chronic pyelonephritis
49:214	76	M	20.25	5.15	Metastatic tumor	Neoplasm of urinary bladder
50:120	61	M	19.50	5.85	Metastatic tumor	Carcinoma of esophagus
49:220	46	M	13.75	19.50	Hyperemic kidney	Cervical myelopathy
49:230	21	F	18.88	22.05	Moderate cloudy swelling	Tetralogy of Fallot
50:41	75	M	24.56	21.80	Severe cloudy swelling	Carcinoma of esophagus
49:238	50	M	17.20	17.20	Nephrosis	Strychnine poisoning
50:75	41	M	28.75	23.80	Nephrosis	Generalized lymphadenopathy
50:109	39	F	18.25	21.20	Cholemic nephrosis	Generalized lymphadenopathy
50:114	76	M	18.70	21.75	Pyelonephrosis and hydronephrosis	Carcinoma of uterine stump
50:119	70	M	18.21	19.75	Hydronephrosis	Nodular hyperplasia of prostate
50:211	49	F	15.85	16.25	Cholemic nephrosis	Cholecystectomy
49:242	42	F	5.85	23.28	Necrotizing arteries	Necrotizing arteries of kidneys and lungs
50:36	77	M	10.51	12.51	Arteriolar and arterial nephrosclerosis	Endocarditis
50:44	80	M	16.82	16.72	Arterial nephrosclerosis	Generalized arteriosclerosis
50:55	58	M	28.67	29.15	Arteriolar nephrosclerosis	Chronic tuberculosis
50:61	57	F	24.56	26.72	Arteriolar nephrosclerosis	Generalized arteriosclerosis
50:101	31	F	22.50	18.75	Arterial and arteriolar nephrosclerosis	Malignant hypertension
50:103	66	F	12.50	18.79	Arteriolar nephrosclerosis	Essential hypertension
50:110	67	M	11.50	15.20	Arteriolar nephrosclerosis	Myeloma
50:117	87	F	17.50	20.50	Arterial and arteriolar nephrosclerosis	Generalized arteriosclerosis
50:122	98	M	16.50	19.50	Arterial and arteriolar nephrosclerosis	Generalized arteriosclerosis
50:124	70	M	22.50	18.50	Arterial and arteriolar nephrosclerosis	Chronic myocardial failure
50:249	52	M	15.20	1.75	Arterial and arteriolar nephrosclerosis	Diabetes mellitus
50:48	53	M	14.52	3.20	Arterial and arteriolar nephrosclerosis	Diabetes mellitus
50:79	3	F	73.70	63.62	Adenomyosarcoma	Renal adenomyosarcoma
50:81	50	M	18.75	11.50	Infarctions	Pulmonary granulomas
50:205	42	M	17.50	29.50	Chronic glomerulonephritis	Chronic glomerulonephritis
49:232	61	M	15.80	16.81	Retention cysts	Carcinoma of lung
50:46	71	F	23.10	17.85	Chronic glomerulonephritis	Adenocarcinoma of skull bones
50:207	60	F	18.75	14.09	Fibroma and arteriolar nephrosclerosis	Generalized arteriosclerosis

* The concentrations of copper are expressed in milligrams per 100 gm. of fresh tissue.

In the 56 cases listed in Table 3, showing evidence of hepatic pathological changes but not of renal changes, the copper content was in general slightly lower in cases of chronic passive congestion. Hepatic neoplasms were found to have a distinct effect on the copper levels of both the liver and the kidneys. In a series of 17 subjects in whom hepatic neoplasms were found, 16 had low levels of hepatic copper while only 2 had low levels of renal copper. Of these 17, 7 had

TABLE 3.—*Concentrations of Copper in Liver and Kidney When There Was Morphological Evidence of Abnormality in the Liver While the Kidney Was Essentially Normal*

Autopsy	Age	Sex	Kidney*	Liver*	Morphologic Changes in Liver	Anatomic Diagnosis
49:219	39	F	17.88	3.83	Tumor type and source undetermined	Glioblastoma multiforme
49:223	59	F	24.00	12.79	Metastatic carcinoma	Carcinoma of sigmoid
49:234	66	M	12.20	6.70	Metastatic carcinoma	Carcinoma of prostate
49:241	69	F	30.67	12.92	Primary carcinoma	Hepatic and primary carcinoma
49:248	67	M	15.75	8.84	Metastatic carcinoma	Carcinoma of body and tail of pancreas
49:251	58	M	20.50	12.72	Hemangioma	Chronic endocarditis
49:257	45	M	14.12	1.61	Metastatic carcinoma	Carcinoma of tail of pancreas
49:260	5 mo.	F	12.15	2.50	Metastatic carcinoma	Neuroblastoma of left adrenal
50:08	42	M	2.40	1.98	Metastatic carcinoma	Carcinomatosis of pancreas
50:56	68	M	29.68	22.80	Metastatic carcinoma	Carcinogenic metastases to liver and spleen
50:60	44	F	28.75	10.35	Metastatic carcinoma	Chorioepithelioma of uterus
50:74	60	M	29.29	7.67	Metastatic carcinoma	Carcinoma of pancreas
50:94	37	M	14.75	7.50	Metastatic carcinoma	Carcinoma of head of pancreas
50:120	61	M	10.50	5.85	Metastatic carcinoma	Carcinoma of esophagus
50:214	63	M	1.50	0.833	Metastatic carcinoma	Adenocarcinoma of ileocecal junction
50:252	49	M	4.50	0.980	Metastatic carcinoma	Carcinoma of rectum
50:32	68	F	26.55	3.51	Cavernous hemangioma	Aplastic anemia
49:218	9	M	14.74	12.06	Chronic passive congestion	Rheumatic pericarditis
49:224	3.5 mo.	M	6.36	4.43	Chronic passive congestion (severe)	Stenosis of distal colon
49:239	80	M	22.50	17.63	Chronic passive congestion	Uremia
49:244	72	M	25.87	12.18	Chronic passive congestion	Carcinoma of larynx
49:249	59	M	18.50	12.50	Chronic passive congestion	Generalized arteriosclerosis
49:250	26 days	M	38.50	12.50	Chronic passive congestion	Bronchopneumonia
49:262	80	M	27.45	21.80	Chronic passive congestion	Generalized arteriosclerosis
50:01	39	F	16.42	13.50	Chronic passive congestion	Congenital heart disease
50:02	45	M	36.42	25.42	Chronic passive congestion	Generalized arteriosclerosis
50:10	12	F	32.50	14.82	Chronic passive congestion	Poliomyelitis
50:35	47	F	23.72	22.80	Chronic passive congestion	Operational wound
50:37	52	F	19.09	22.14	Chronic passive congestion	Cholelithiasis
50:39	39	M	15.19	34.23	Chronic passive congestion	Intracranial neoplasm
50:47	44	F	16.71	21.72	Chronic passive congestion	Thrombosis of pulmonary artery
50:52	74	M	32.86	28.78	Chronic passive congestion	Pericarditis
50:63	24	M	13.78	14.25	Chronic passive congestion	Myocardial infarction
50:66	51	F	15.47	22.23	Chronic passive congestion	Generalized arteriosclerosis
50:69	65	M	26.28	24.83	Chronic passive congestion	Coronary arteriosclerosis
50:73	87	F	18.67	27.56	Chronic passive congestion	Generalized arteriosclerosis
50:107	71	M	31.50	16.80	Chronic passive congestion	Extradural neoplasm
50:84	68	M	15.47	22.09	Liver cirrhosis	Calcified aortic stenosis
49:245	15	F	15.06	22.68	Liver cirrhosis	Liver cirrhosis
49:260	68	M	27.58	21.32	Liver cirrhosis	Dilatation of heart
50:00	74	M	32.16	22.81	Biliary cirrhosis	Carcinoma of prostate
50:27	49	M	17.12	22.36	Early hepatic cirrhosis	Tumor of right lung
49:215	29	F	14.08	11.50	Chronic passive congestion	Neoplasm of lung vessels
49:217	68	F	16.32	22.80	Chronic passive congestion	Emphysema
50:03	21	F	13.78	22.50	Fatty degeneration	Contusion of left temporal lobe
50:04	42	M	15.30	20.50	Fatty degeneration	Tuberculous meningitis
50:29	72	F	29.75	20.18	Fatty degeneration	Gastric ulcer
50:78	45	M	18.50	19.58	Acute fatty degeneration	Lead poisoning
50:95	..	M	22.08	25.68	Moderate fatty degeneration	Ulcerative colitis
49:228	85	F	13.40	22.02	Marked cloudy swelling	Generalized arteriosclerosis
49:248	67	M	13.50	18.75	Mild cloudy swelling	Carcinoma of rectum
49:250	59	M	18.91	13.51	Hemochromatosis	Diverticulitis
50:19	21	F	26.85	26.58	Severe hemosiderosis	Hemolytic anemia
50:84	37	F	11.50	22.50	Hepatomegaly	Monocytic leukemia
50:100	50	F	26.78	18.76	Amyloidosis	Amyloidosis of spleen and liver
50:121	4	M	13.50	18.75	Miliary tuberculosis	Pulmonary tuberculosis

* The concentrations of copper are expressed in milligrams per 100 gm. of fresh tissue.

hepatic copper levels varying from 0.833 to 3.83 mg. per 100 gm. of tissue, 5 had levels varying from 5.35 to 8.84 mg., while 4 had concentrations varying from 10.35 to 12.92 mg. per 100 gm. of tissue.

In the necropsies listed in Table 4, showing both renal and hepatic pathological changes, no significant changes in the renal and hepatic copper levels were found. An interesting finding was in Necropsy 49:227 of a 30-month-old boy having a hypoplastic kidney. The copper content of the right hypoplastic kidney was 4.08, while the left kidney contained 10.48 mg. per 100 gm. of tissue.

TABLE 4.—*Concentrations of Copper in Liver and Kidney When There Was Morphological Evidence of Abnormality in Both Liver and Kidney*

					Morphologic Changes		
Autopsy	Age	Sex	Kidney*	Liver*	Kidney	Liver	Anatomic Diagnosis
49:209	51	F	14.91	16.75	Arterial and arteriolar nephrosclerosis	Moderate fatty infiltration	Mitral stenosis
49:227	2½ mo.	M	R. 4.08 L. 10.48	12.69	Hypoplasia right kidney	Biliary cirrhosis	Biliary cirrhosis
49:228	85	F	13.40	22.02	Arteriolar nephrosclerosis	Simple cyst	Generalized arteriosclerosis
49:229	31	F	15.76	18.75	Severe arteriolar nephrosclerosis	Marked cloudy swelling	Hypertrophy of heart
49:231	10	F	6.15	16.69	Marked cloudy swelling	Marked cloudy swelling	Poliomyelitis
49:232	65	F	12.32	19.52	Marked cloudy swelling	Marked cloudy swelling	Carcinoma of thyroid
49:235	66	M	16.87	19.67	Marked cloudy swelling	Marked cloudy swelling	Cerebrovascular accident
49:236	68	M	18.74	10.70	Renal calculus	Fatty degeneration	Calculus of kidney
49:243	44	M	22.78	22.50	Congestion	Chronic passive congestion	Uremia
49:267	44	F	16.52	10.42	Arterial nephrosclerosis	Chronic passive congestion	Essential hypertension
49:270	66	F	12.75	17.80	Fatty infiltration	Fatty infiltration	Carcinoma of stomach

* The concentrations of copper are expressed in milligrams per 100 gm. of fresh tissue.

SUMMARY

It was found by chemical analysis of 148 specimens of renal and of hepatic tissues obtained at autopsy that certain pathological conditions have a distinct effect on the copper levels in these organs. A series of 33 subjects varying in ages from 17 to 80 years, in whose case no pathological changes were noted in the kidneys and the liver, showed the copper content of the liver ranged from 10.88 to 28.80 and that of the renal tissue from 17.75 to 30.76 mg. per 100 mg. of fresh tissue. In the first 12 necropsies mentioned in Table 1, the copper levels were found to be slightly lower than the normal limits for adults. Persons with hepatic neoplasms were found to have a low hepatic copper content, while 2 who had metastatic renal neoplasms were found to have normal copper levels in the kidneys, but the hepatic copper levels were below 5.85 mg. per 100 gm. of tissue. In a series of 17 persons in whom hepatic neoplasms were found, there were 16 who had low levels of hepatic copper, while only 2 had low levels of copper in the kidneys. In the instance of reticulum cell sarcoma without evidence of either renal or hepatic

disease, the renal copper content was within normal limits, but the liver was found to contain only 3.5 mg. per 100 gm. of tissue. An unusually high copper concentration was found in both liver and kidney in a case of renal adenomyosarcoma.

Diabetes mellitus was another disease in which low hepatic copper levels were found; however, the renal copper levels were within normal limits. In a series of 5 cases of hepatic cirrhosis and one case of hemochromatosis, the kidney and liver copper levels were found to be within normal limits. In a case of necrotizing arteritis, the kidney copper content was 5.85 mg. per 100 gm. of tissue, while the hepatic copper level was within normal limits.

These findings suggest that renal and hepatic copper levels of 20 to 30 mg. for adults and 14 to 20 mg. per 100 gm. of tissue for children constitute a normal range. In certain pathological conditions, as mentioned above, significant notable changes in the distribution of copper were observed in certain organs of the body.

Dr. D. M. Angevine and other members of the Department of Pathology cooperated in furnishing the necropsy material.

EFFECT OF COPPER ON HEPATIC TUMORS PRODUCED BY 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE

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IT HAS been previously reported by us [1] that the copper levels in the liver and the kidney are significantly altered by the presence of neoplasms. Sixteen of 17 persons in whom necropsies showed evidence of renal or hepatic tumors were found to have a low copper concentration in the liver while only 2 of these 17 had a low copper content in the kidney.

Sharpless [2] reported that in rats increases of 0.15, 0.3, and 0.5% in dietary copper prolonged the induction period of hepatic tumors produced by *p*-dimethylaminoazobenzene. He observed that the copper did not prevent the development of the tumors but the induction period, determined by either palpation or by the period necessary to kill the animals, was increased from 25 to 50%.

Since certain types of neoplasms significantly altered the copper content in human livers and in several cases in the kidney, it was of interest to determine whether or not the carcinogen, 3′-methyl-4-dimethylaminoazobenzene, may have a similar effect.

MATERIALS AND METHOD

Ninety male albino Sprague-Dawley rats (170 to 250 gm.) were employed for this work. A group of 10 rats was fed the semisynthetic diet suggested by Miller and Miller,[3] which contains 0.098% copper. Two groups of 10 rats each received the diet supplemented, respectively, with 0.25 and 0.5% copper. A fourth group, of 30 rats, received the diet supplemented with 0.059% 3′-methyl-4-dimethylaminoazobenzene, while a fifth group, of 20 rats, received the diet supplemented with 0.059% 3′-methyl-4-dimethylaminoazobenzene and 0.25% copper. A sixth group,

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3. Miller, J. A., and Miller, E. C.: The Carcinogenicity of Certain Derivatives of *p*-Dimethylaminoazobenzene in the Rat, J. Exper. Med. **87**:139, 1948.

of 10 rats, received the diet supplemented with 0.059% 3'-methyl-4-dimethylaminoazobenzene and 0.5% copper. The rats were maintained on these diets for 180 days and then killed.

The liver specimens for copper analysis were taken from areas showing no gross evidence of tumor or from areas with the minimal neoplastic involvement. Approximately 3 gm. of hepatic and 1 gm. of renal tissue were used for the determinations. The method employed for the determination of the copper was the same as that previously described.[1]

RESULTS

The pertinent data are presented in the accompanying table. The addition of 0.25% copper to the diet increased the copper content of the kidney and the liver approximately fivefold and the animals presented no toxic symptoms and appeared to be normal. The diet which was supplemented with 0.5% copper was found to be too toxic, since only one of the 10 animals survived the 180-day period. Of the 30 rats in Group 4 fed the carcinogen, 22 survived the 180-day test period. Of these

Concentrations of Copper, Expressed in Milligrams per 100 Gm. of Fresh Tissue, in Kidneys and Livers of Rats Fed Supplemental Copper and 3'-Methyl-4-Dimethylaminoazobenzene

Supplement to Diet	Animals	Number Surviving 180 Days	Copper Content of Animals Surviving 180 Days *	
			Kidney	Liver
Group 1				
None	10	10	0.850- 2.15	1.70 - 3.15
Group 2				
0.25% copper	10	10	5.85 -17.65	4.09 -14.19
Group 3				
0.5% copper	10	1	8.67	16.89
Group 4				
0.059% 3'4 DAB	30	22	0.608- 3.15	0.975- 2.15
Group 5				
0.059% 3'4 DAB and 0.25% copper	20	15	4.41 -12.67	3.85 -15.08
Group 6				
0.059% 3'4 DAB and 0.5% copper	10	0	1.50 -13.50 †	2.65 -14.01

* The values listed represent the range from the lowest to the highest copper concentration found in all the animals.

† The range is that found for the 10 animals which died between 10 and 100 days.

22 animals, 19, or 86.3%, had hepatomas. Of the eight rats in Group 4 which did not survive the 180-day period, five died between 90 and 100 days and these, when autopsies were made, had hepatomas, which makes an incidence of 62.5%. Fifteen of the 20 rats in Group 5 receiving food containing 0.059% 3'-methyl-4-dimethylaminoazobenzene and 0.25% copper survived the 180-day period, 10 of which had hepatomas, which makes an incidence of 66.6%. Of the five animals in Group 5 which died before the termination of the 180-day test period, two died after 95 to 100 days and both of these had hepatomas, making an incidence of 40%. Analysis of the hepatic and renal tissues of the 22 animals in Group 4 which survived the test period showed that the hepatic and renal copper levels were within the normal limits. The renal copper levels of the eight animals in Group 4 which did not survive the test period were also within normal limits, but the hepatic copper levels of the five animals with hepatomas were found to be low. Concentrations of 0.476, 0.480, 0.496, 0.530, and 0.598 mg. of copper per 100 gm. of fresh tissue were found in the livers of these five animals with hepatomas.

The renal and hepatic copper levels of all the animals of Group 5 surviving the 180-day test period were found to be within the range found for those animals in Group 2 which received the diet supplemented by 0.25% copper. The two rats in Group 5 which did not survive the test period but died between 95 and 100 days with neoplasms in the liver were found to have low hepatic copper levels. Copper concentrations of 0.532 and 0.537 mg. per 100 gm. of fresh tissue were found in the livers of these two animals. The renal and hepatic copper levels of the animals of Group 5 which survived the test period but had no hepatomas were found to be within the range of those animals in Group 2 fed the same diet but without the carcinogen.

None of the animals fed the carcinogen supplemented with 0.5% copper survived more than 100 days; five of the animals died after 85 to 100 days, and none of the animals had any microscopic or gross evidence of a hepatoma. The copper content of the liver was significantly lowered in five of these animals, since they had hepatic copper levels below 5.85 mg. per 100 gm. of fresh tissue, which was the lowest concentration of copper found in the livers of the animals fed the basal diet supplemented with 0.25% copper.

COMMENT

The results indicate that copper added to the diets of rats fed the carcinogen 3′-4-dimethylaminoazobenzene exerts an inhibitory influence on the tumor development. The percentage incidence of hepatomas in the animals supplied the carcinogenic diet and surviving the 180-day test period was 86.3, while the percentage incidence of hepatomas in animals surviving this test period but fed the carcinogen supplemented with 0.25% copper was 66.6. Moreover, the percentage incidence of hepatomas in the animals dying after 90 to 100 days was 62.5 with the carcinogenic diet, and only 40 in the animals fed the carcinogen supplemented with 0.25% copper. Although the addition of 0.5% copper to the carcinogenic diet was too toxic, it appears significant that in all the animals which died between 85 and 100 days no evidence of hepatoma was found.

SUMMARY

These findings indicate that a concentration of 0.25% copper added to the diets of rats fed 3′-methyl-4-dimethylaminoazobenzene exerts an inhibitory influence on the tumor development.

Subnormal hepatic copper levels were found in some animals with hepatomas induced by feeding with 3′-methyl-4-dimethylaminoazobenzene. This constitutes experimental evidence that the presence of neoplasms can significantly decrease the copper concentration in the liver.

HISTOCHEMICAL ANALYSIS OF PATHOLOGICAL MINERAL DEPOSITS AT VARIOUS SITES

With Discussion of Methods Used

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THERE are numerous sites in the body where mineralization occurs under pathological conditions. Many deposits are large enough so that their consistency leaves little doubt that calcium is a prominent constituent. There are others, however, of microscopic size. The affinity of these foci for mordanted hematoxylin as employed in routine stains is commonly regarded as evidence that they consist of a salt of calcium. The question whether iron is present in them has been debated since Gierke [1] first stated that iron was absorbed by the substrate prior to the actual precipitation of calcium salts. Hueck [2] maintained that this iron might have been derived from impurities in the fixing fluids. He also agreed with Noesseke [3] that it could have diffused into the fixing solution from the adjacent tissues, since he could demonstrate it only after tissues had been in fixatives for some time. W. G. MacCallum [4] believed rather that the fixatives simply liberated the iron from some combination which at first had prevented its giving the Prussian-blue reaction. He demonstrated iron regularly in foci of pathological calcification just as occurs also in the line of normal ossification of the subperiosteal growing bone.

Cameron[5] carried out an extensive study of this problem, devoting much attention to the technical aspects. He concluded that oxidized hematoxylin (hematein) did not reveal calcium but areas in which there were changes favorable to the deposition of calcium salts; that the reaction obtained in these areas depended partly on the presence of iron but mostly on a peculiar ground substance which was normally present in bone and cartilage and also at sites of pathological calcification, and that the von Kossa reaction in these deposits depended on the anion rather than on calcium or other metal. He also demonstrated "in a fair proportion of cases" the association of iron and calcium, but inasmuch as this could more readily be done

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1. Gierke, E.: Arch. path. Anat. **167**:318, 1902.
2. Hueck, W.: Zentralbl. f. allg. Path. **19**:774, 1908.
3. Noesseke, H.: Zentralbl. allg. Path. **20**:56, 1909.
4. MacCallum, W. G.: A Text-Book of Pathology, ed. 5, Philadelphia, W. B. Saunders Company, 1932.
5. Cameron, G. R.: J. Path. & Bact. **33**:929, 1930.

in tissue that had lain for prolonged periods in fixative he cautioned about the source of the iron. For a more extensive coverage of the literature the reader is referred to Cameron.[5]

The present report is a study of the occurrence of iron and calcium as well as of the acidic components, carbonate and phosphate, in pathological mineral deposits at various sites—for example: the walls of blood vessels in the brain and elsewhere; interstitially in the choroid plexus, the brain, the kidney, and other organs, and intracellulary in myocardial fibers. The results obtained frequently do not differ from those of certain of the previous investigators, but the presentation appears warranted since direct methods have been employed in contrast to those often used by others, which have either lacked specificity or have been erroneously interpreted.

METHODS

The material was mostly derived from the autopsies at the New Haven Hospital, one to six hours after death. A few specimens were obtained from experimental animals (dogs treated with viosterol) of Drs. W. B. McAllister and L. L. Waters.[6] The presence of ferric iron was detected in sections fixed in 4% formaldehyde solution U. S. P., preferably buffered at pH 7,[7] by the Prussian-blue reaction (potassium ferrocyanide and hydrochloric acid) slightly modified from that of Lišon.[8] This consisted of a 60-minute exposure (solution renewed after 30 minutes) of the section to equal parts of 2% hydrochloride acid and 2% potassium ferrocyanide. Ferrous iron has been detected with 20% ferrocyanide solution to which has been added an equal volume of 1% hydrochloric acid but without pretreatment with ammonium sulfide as in the Turnbull-blue reaction. No counterstain was employed in either of these. Both ferrocyanide and ferricyanide were used in certain instances without hydrochloric acid. Microincineration and subsequent examination by dark-field illumination[9] were used frequently for confirmation-studies of the presence of iron. Calcium was detected in sections fixed in 4% plain or buffered formaldehyde solution U. S. P. by two methods. The first was by the formation of an orange or reddish-orange lake when the section was exposed to a 1% aqueous solution of alizarin red S; staining was complete in a few minutes and was followed by washing in water (not differentiated in acids, as this removes small deposits of calcium), dehydrated, and mounted in a synthetic mounting medium (Harleco). Alizarin formed purple lakes with the inorganic iron found in most of the deposits to be described (thus differing from hemosiderin). The definitive test for inorganic calcium was given by the formation of characteristic crystals of hydrated calcium sulfate ($CaSO_4 \cdot 2H_2O$). These form as long, slender, colorless needles that become thin prisms with truncated ends and finally masses of thin, piled-up plates, commonly twinned.[10] These develop from the actual deposit under investigation after a microdrop of dilute (approximately 5%) sulfuric acid has been applied directly to the area in question in a deparaffinized section under microscopic observation. The crystals shown in Figure *3D* are an example of crystals of hydrated calcium sulfate developing on spicules of bone. Microincinerated sections were as a rule not as helpful, since under these conditions all the combined ("masked") calcium in addition was available for the reaction.

Inorganic phosphate was detected in sections fixed in 4% formaldehyde solution U. S. P. by the molybdenum-blue reaction slightly modified from Feigl[11] and Serra and Queiroz-Lopes.[12]

6. McAllister, W. B., and Waters, L. L.: Yale J. Biol. & Med. **22**:651, 1950; other investigations to be published.

7. Lillie, R. D.: Histopathologic Technic, Philadelphia, The Blakiston Company, 1948.

8. Lišon, L.: Histochimie animale: Méthodes et Problèmes, Paris, Gauthier-Villars, 1936.

9. Policard, A.: J. Roy. Micr. Soc. **62**:25, 1942.

10. Chamot, E. M., and Mason, A. W.: Handbook of Chemical Microscopy, New York, John Wiley & Sons, 1947.

11. Feigl, F.: Qualitative Analysis by Spot Tests, Inorganic and Organic Applications, New York, Elsevier Pub. Co., 1946.

12. Serra, J. A., and Queiroz-Lopes, A.: Portugaliae acta biol. **1**:111, 1945.

Helly's fluid [13] could not be used because the reaction was blocked presumably by the combination of the mercury and the phosphate. A few drops of 5% ammonium molybdate were placed on the deparaffinated section; an equal amount of 1% nitric acid was then added. After five minutes the section was thoroughly washed and subsequently covered with benzidine solution (0.05 gm. of benzidine base and 10 ml. glacial acetic acid made up to 100 ml. of H_2O), and after one minute the section was immersed in or flooded with saturated aqueous sodium acetate. The reaction was evaluated immediately, since the blue color (formed from both molybdenum blue and oxidized benzidine, the two constituting an oxidation-reduction system) fades, and also the localization becomes less precise after several hours.

Carbonate was detected presumptively by the gas generated from the site on the deparaffinized slide on which a small drop of dilute acid had been applied. The gas bubbles could be positively identified as carbon dioxide as follows: The area was first covered with a drop of mineral oil, and then a microdrop of acid was applied to the deposit in the section beneath it; after the gas bubble had formed, the acid drop was jarred or pushed aside and replaced by a drop of saturated barium hydroxide solution in contact with the gas bubble; a precipitate slowly formed at the interface of the gas and the barium hydroxide solution; the use of lead acetate alone would have been simpler but was not found successful, as the amount of carbonate liberated was apparently too small.

Basophilia has been determined by staining for about two hours with dilute methylene blue (10^{-4} M) buffered at pH 7.6, washing the slide briefly in water, drying in air, placing in xylene, and mounting.

In a few instances lipids were sought for. Frozen sections were stained in Sudan IV in glycol.[14]

Most of the deposits or regions studied were heavily stained by hematoxylin in the routine hematoxylin-eosin preparations. Since the hematoxylin as so used (Harris' hematoxylin) is in the oxidized state ("hematein") and already combined with a mordant, it was thought of interest to observe the reaction of these regions with unoxidized hematoxylin,[15] since this reacts with inorganic iron.[16]

DISCUSSION OF CERTAIN METHODS AND THEIR INTERPRETATION, BASED ON CONTROL STUDIES

Demonstration of Iron.—Four points bear comment: 1. The dilute hydrochloric acid-ferrocyanide solution (1% concentration of each in the final solution) recommended by Lišon was employed rather than the solutions using a more concentrated acid since the highly soluble iron that was found in some of the small deposits could not be detected with the latter.[16]

2. It was apparent that exposing the material to a fixative was not necessary in order to obtain a positive reaction for iron, and thus manifestly that iron was not necessarily derived from the fixing solutions, since a positive reaction has been obtained with ferrocyanide and hydrochloric acid where this was tried in deposits in fresh, unfixed material from the choroid plexus and the aorta.

3. It had been observed in previous work [16] that the soluble iron in these deposits, in contrast to the iron of hemosiderin, possessed an affinity for pure hematoxylin as well as for the mordanted hematein commonly used in staining. It had also been observed that hemosiderin treated with dilute ammonium sulfide was reactable with hematoxylin, but whether this was due entirely to the con-

13. Helly's fluid is a modification of Zenker's solution in which, instead of glacial acetic acid, formaldehyde solution U. S. P. is added in the concentration of 5%.

14. Chiffelle, T. L., and Putt, F. A.: Stain Technol. **26**:51, 1950.

15. Macallum, A. B.: J. Physiol. **22**:92, 1896.

16. Bunting, H.: Stain Technol. **24**:109, 1949.

version of ferric to ferrous iron or to a hydrolytic effect was not certain. The present experiments make the latter explanation seem probable since in six examples hematoxylin lakes have been formed when the only iron detectable was ferric.

Control studies employing an undecalcified section of a developing rib demonstrated that iron was present in the immediately subperiosteal cortical bone (as described by W. G. MacCallum [4]). Pure hematoxylin stained this line alone, while mordanted Harris' (or Bullard's) hematoxylin (hematein) stained in addition other noncellular material in the trabeculae—for example, the cement lines. The solid, calcified portion of the trabeculae was essentially unstained, however, with both types of hematoxylin. When the section was exposed to 10% nitric acid, iron, as well as calcium, was removed, and there was nothing stained after this treatment by pure hematoxylin, while the picture was unchanged when the section was stained with mordanted hematoxylin (hematein). The same observations have been made on the calcium-iron deposits in the walls of cerebral arteries. Acid removes essentially everything stainable with pure hematoxylin but does not alter that which has an affinity for the mordanted oxidized dye. It is apparent that the calcium itself is not stained by either pure or mordanted hematoxylin. Readily soluble iron is probably responsible for the staining of such sites with pure hematoxylin, but other factors must account for the staining with the mordanted hematoxylin of ordinary preparations. Other observations reported below support this conclusion.

4. The iron is these deposits is at times very soluble: The iron in some sites —for example, the choroid plexus granules—was removed by 30 minutes' soaking in doubly distilled water that had been standing in air, but not after being similarly exposed to a sample that had been freshly boiled.

Demonstration of Calcium.—The alizarin-red-S method for calcium is known not to be very sensitive. Lišon [8] recommended the galloformate method of Cretin as being superior. This has not been successfully applied in our laboratory. The formation of crystals of calcium sulfate has proved more useful than alizarin-red-S for these deposits, since the deeper color (purple) of the combination of alizarin and iron can often obscure the orange-red calcium lake.

Orange-red crystals form on the surface of and within the granular calcium deposits or from solutions of a calcium salt when either is in contact with alizarin. These crystals are birefringent in the polarizing microscope. It is suggested that they represent the calcium-alizarinate combination. No recognizable crystals have been observed on the iron-containing deposits, although these are stained purple or deep blue by the formation of the iron-lake.

The differential extraction by oxalic acid and hydrochloric acid has been occasionally used [17] for separating iron and calcium in deposits. This has not been employed here, since it was observed that oxalic acid (10% for 30 minutes) was capable of removing not only the iron but also the calcium when it was present in trace amounts.

The von Kossa reaction which has commonly been used to detect calcium deposits depends primarily on the silver precipitated from a silver nitrate solution

17. Hurst, E. W.: J. Path. & Bact. **29**:65, 1926.

as either phosphate, carbonate, or some other insoluble salt rather than on the calcium alone. It has not been employed in this study, since it would not contribute any information of histochemical significance.

RESULTS

Representative data are presented in the accompanying table. They will be summarized below according to the site and the type of deposit.

Blood Vessels.—The small arteries and arterioles of the brain have long been known to contain deposits that are heavily stained by hematoxylin. This staining has been thought to be due to the presence of iron by some investigators and to

Representative Reactions Obtained in Various Mineralized Deposits *

	Deposits in Arterial Walls				Interstitial Deposits					Focal Myocardial Mineralization	
	Arteriosclerotic plaque of aorta	Arteriosclerotic plaque of coronary artery	Wall of "calcified" cerebral artery	Wall of intrasplenic arteriole, in chronic hemolytic anemia	"Calcified" intracerebral granules	Choroid plexus granules		Granules in meningioma	Granules in kidney	"Calcified" fibers—human	"Calcified" fibers—dog
Preparation { fresh	+	..	..	..	..	+	..	..	..	..	..
Preparation { fixed	..	+	+	+	+	..	+	+	+	+	+
Reaction of deposit:											
Inorganic ferric iron	+	+	+	+	+	+	+	+	+	+	+
Inorganic ferrous iron	..	+	+	+	+	+	+	+	—	+	+
Inorganic calcium	+	+	+	—	+	+	+	+	+	—	+
Inorganic phosphate	+	+	+	+	+	+	+	+	+	+	+
Presumptive carbonate	+	+	+	—	+	+	+	+	+	—	+
Hematoxylin lake (MacCallum)	+	+	+	+	+	+	+	+	+	+	+
Mordanted-hematein lake (Harris')	+	+	+	+	+	+	+	+	+	+	+
Alizarin lake { purple	..	+	+	+	+	..	..	..	..	+	+
Alizarin lake { orange	+	+	+	..	+	+	+	+	+	..	+
Basophilia (methylene blue)	..	+	+	+	+	+	+	—	±	+	+

* A complete listing of numbers and sites is found in the text. The chemically significant reactions are grouped together. "Presumptive carbonate" signifies that gas bubbles evolved from the deposits on the addition of dilute acid. Lakes formed by the deposits with pure hematoxylin and with the oxidized hematoxylin (hematein) as usually employed, mordanted with iron alum (Harris'), are noted, along with the color of the lake obtained with alizarin red S. Basophilia of the deposits is noted by their affinity for methylene blue.

the presence of calcium by others. Most of the recent work has indicated that both elements are present. Our studies are confirmatory of the last finding. The vessels in sections from 11 brains have been examined (Fig. 1). A strong reaction for iron was observed uniformly throughout the deposit in the arterial wall. The iron deposits encountered reacted with both ferrocyanide and ferricyanide and hence contained both ferric and ferrous components. One cannot conclude that the iron was in this state in the living or even in the fresh tissue; suffice it to say that the valence of the iron here must be very labile in contrast to that in hemosiderin, which is always ferric, as observed. Calcium was shown to be present in most instances by the formation of crystals of hydrated calcium sulfate. The

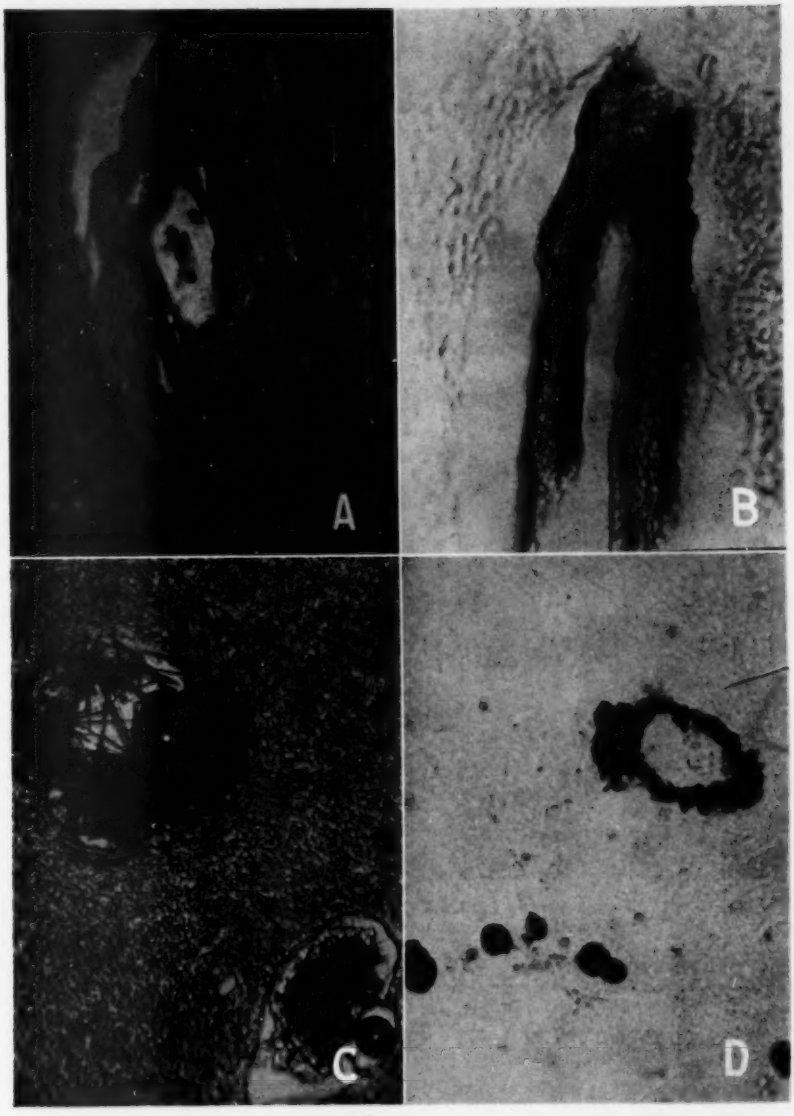

Fig. 1.—*A*, cerebral arteriole stained with dilute methylene blue. The basophilia of the granular deposit in the subintima with encroachment on the media is evident. × 380.

B, inorganic phosphate demonstrated in the subintima and the adventitia of a cerebral arteriole. The media contains relatively little. The greenish blue color of the reaction photographs black. × 380.

C, wet preparation obtained by adding a microdrop of dilute sulfuric acid to a deparaffinated section. Bubbles of carbon dioxide were liberated from the deposits in the walls of cerebral arterioles on addition of acid. Crystals of hydrated calcium sulfate are also evident, originating from the deposits. × 135.

D, Prussian-blue reaction in the deposit in the wall of a cerebral arteriole and in small interstitial granules. No counterstain; × 380.

orange color of the alizarin-calcium lake was seen, but it was often obscured by the deeper purple of the alizarin-iron lake. These deposits were stained by both the mordanted oxidized hematoxylin and the pure hematoxylin. They were rather strongly basophilic with methylene blue, and both phosphate and carbonte could be demonstrated in most examples.

The location of the deposit, as revealed by the detection of the various constituents, basic as well as acidic, was always the same. When the deposit was small in amount, it was usually confined to the subintima in the region of the elastica, or if larger, it involved in addition the adventitia and outer media, with as a rule some atrophy of the smooth muscle of the media. In the extreme instances the media was entirely replaced and the lumen reduced or obliterated by the deposit. No lipid could be demonstrated by Sudan-IV staining in any of the smaller

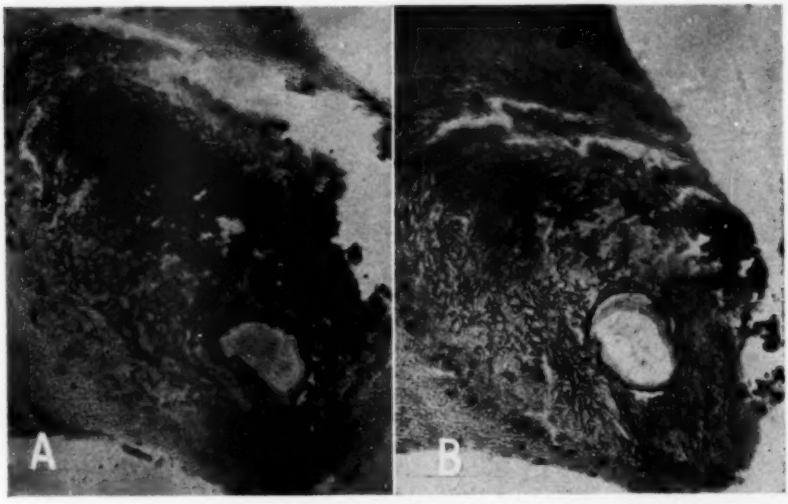

Fig. 2.—*A*, small splenic artery in chronic hemolytic anemia stained with potassium ferrocyanide and hydrochloric acid. Note the reaction in the elastica as well as in the deposit in the rest of the arterial wall. × 75.

B, same vessel stained with potassium ferricyanide and hydrochloric acid. A positive reaction is obtained in the same sites although it is not as heavy as that in *A*. × 75.

"calcified" vessels in the sections of three brains. Trace amounts were present within phagocytes in some of the larger vessels (2 mm.) but the location bore no necessary relation to the mineral deposit.

In the aorta and vessels elsewhere (coronary, splenic, and uterine arteries, seven examples) the deposits showed in general similar constituents. The reactions for iron, however, were less strongly given than in the cerebral vessels, suggesting a lower concentration in association with calcium. The iron usually appeared at the periphery of the calcium carbonate and phosphate plaque. The affinity of iron for the elastica was occasionally seen. This was observed in small splenic arteries (Fig. 2) in congenital hemolytic icterus, in hemochromatosis

(three examples), and in the vessels in experimental material from dogs, the coronary arteries of which had been damaged by allylamine (Waters [18]) (four examples).

The deposits in the walls of the arteries of the spleen in the instances of excessive iron storage are of interest. No calcium or carbonate was detected in the examples studied, but iron (ferrous and ferric) and phosphate were present, accounting for the affinity shown for hematoxylin and methylene blue.

Granular Deposits in the Interstitial Tissue.—The spherical granules commonly present in the interstitial tissue of the choroid plexus (13 examples studied) and at times within the white matter of the brain (Fig. 1*D*) (10 examples studied) contained as a rule the same constituents as the deposits in the walls of the intracerebral arteries. Frequently there was variation in the staining of individual granules with alizarin, some being orange while others were mainly purple or only purple-rimmed. The same variation was found in the localization of iron (stainable by both ferrocyanide and ferricyanide with hydrochloric acid). Some granules were stained uniformly, while others were stained only at the periphery. All of these granules when unstained were white and only slightly translucent. Another type of granule that differed somewhat was also encountered. It was transparent and glassy in appearance, by contrast, and was commonly larger than the other type when the two were found together. Granules of the second type often were composed of concentric laminations, which undoubtedly accounted for the cross of polarization presented when they were viewed between crossed Nicol prisms. They were orange after alizarin staining, and like the others gave rise to gypsum crystals in the presence of sulfuric acid. Both types of granules contained phosphate and carbonate, but the glassy ones were seldom stained with methylene blue. Iron was not conspicuous in the glassy granules and was confined to the outer rim alone. The rim only was stained by MacCallum's hematoxylin. The interstitial granules of a meningioma (one example studied) gave reactions similar to those of the glassy type described above.

The "calcium deposits" found interstitially in the medulla of the kidney (three examples) and the Schaumann bodies of Boeck's sarcoid (one example) gave reactions the same as those of the nonglassy granules described above.

Myocardium.—In renal insufficiency with or without secondary parathyroid hyperplasia there may at times be groups of myocardial fibers devoid of nuclei that stain heavily with mordanted hematoxylin. The fibers thus affected may be present in small groups of three or four or more. They are commonly referred to as calcified. The material examined (six examples from human autopsies and three from experimental material [6]) have all contained iron stainable with both ferrocyanide and ferricyanide in the presence of hydrochloric acid (Fig. 3*A*, *B*, and *C*). In one instance the reaction with ferrocyanide was obtained directly in the absence of acid. Phosphate has been conspicuous. The iron and phosphate reactions are frequently localized in the myofibrils and even in the cross striations, which may sometimes be irregularly identifiable. These fibers are quite strongly basophilic with methylene blue and stain readily with MacCallum's hematoxylin, as well as with mordanted hematein. The affinity which these fibers showed for hematein and hematoxylin was clearly not due to calcium but probably was to be attributed to the inorganic

18. Waters, L. L.: Am. Heart. J. **35**:212, 1948.

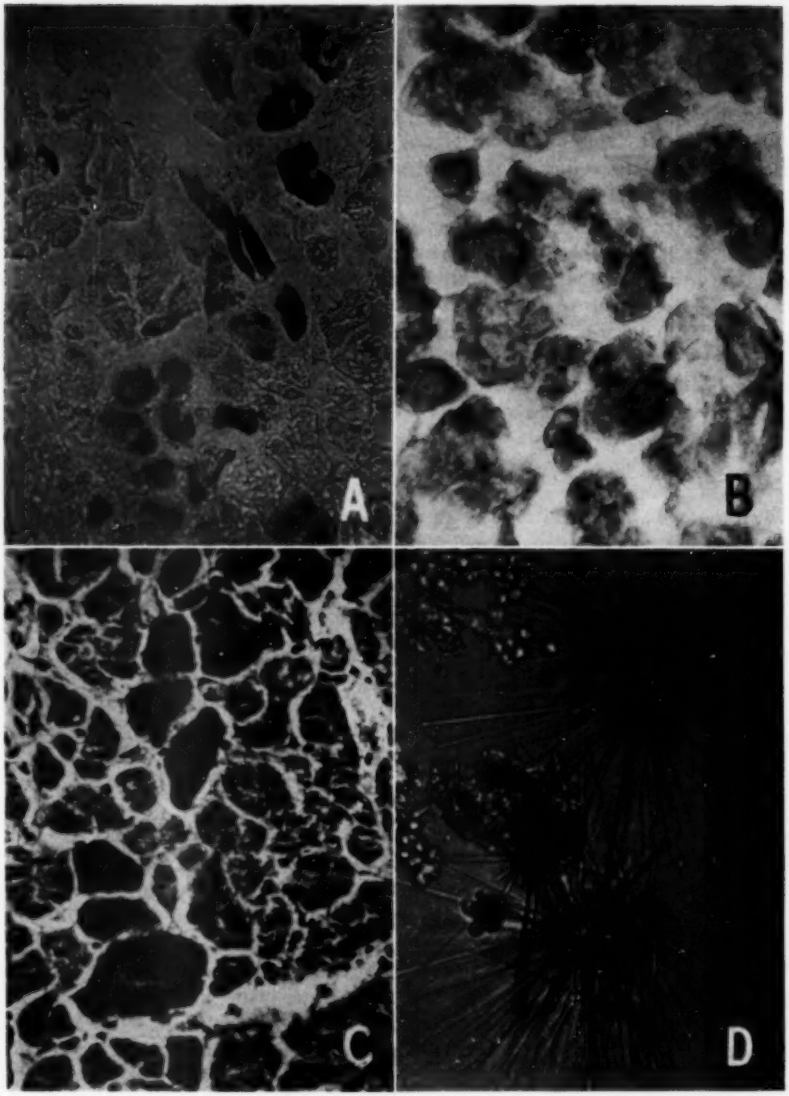

Fig. 3.—*A*, positive reaction obtained in damaged myocardial fibers with potassium ferrocyanide without hydrochloric acid. × 380.

B, inorganic phosphate demonstrated in same fibers as pictured in *A*. × 380.

C, basophilia of the fibers in *A* and *B* demonstrated by their greater affinity for dilute methylene blue than the adjacent unaffected fibers. × 380.

D, characteristic crystals of hydrated calcium sulfate growing from bone spicules in control material on addition of dilute sulfuric acid. × 380.

iron. In the group examined, deposits of calcium and carbonate have been found in one experimental animal, a dog in which the myocardium had been directly damaged.[6] This may well represent a later development of the lesion found in the particular human examples that were studied, since the structure of the muscle cells could no longer be discerned in the midst of the granular calcium deposits.[18a]

COMMENT

This study emphasizes the frequency with which iron is associated with calcium, carbonate, and phosphate in interstitial deposits throughout the body. The derivation of iron by artefact from the fixative, while certainly possible in some instances, has been reasonably well eliminated in others by using fresh tissue in control experiments. The results obtained on the same material after fixation were in no way different. The iron of the deposits differs from that derived from hemoglobin destruction or hemosiderin, which is usually intracellular in location and uniformly ferric. That of the deposits is almost always both ferric and ferrous whether encountered in fresh or in formaldehyde-fixed frozen or paraffin sections. It further differs from hemosiderin by virtue of its greater ease of solubility—for example, in distilled water that has been exposed to the carbon dioxide of the air. This may explain why at times small amounts of iron in these deposits cannot be demonstrated with the usual Prussian-blue methods employing with the ferrocyanide an acid solution more concentrated than that used in this study. Furthermore, a direct Prussian-blue reaction can be obtained occasionally without acid, no hydrolysis being needed. Many of these sites of iron deposition will be obscured if a counterstain is employed.

At the present time there is no ready explanation of the presence of the iron in these deposits. The fact that it occurs only in the outermost layers of some of the granules containing calcium phosphate and carbonate, or in the immediately subperiosteal bone, suggests that here the iron could be derived through an exchange reaction if there were a source of ionized iron as, for example, in the tissue fluid. In the damaged myocardial fibers it would seem likely that iron was combined with phosphate, since neither calcium nor carbonate could be demonstrated. In any event, it can be concluded from these data that the iron of all these deposits is in an inorganic combination and that its actual deposition is controlled by physical or chemical factors probably not under the direct influence of cellular activity.

Many of these deposits are of such common occurrence as to be considered normal with increasing age and apparently do not depend on abnormal serum levels of calcium. There may be local conditions that increase the solubility product of calcium and the principal anion, PO_4. Extracellular alkaline phosphatase has been considered essential for pathological as well as for normal calcium phosphate deposition (Moog [19]; Lorch [20]). Preliminary studies on this material have not thus far revealed the enzyme as interstitially present at the site of the deposit. Local

18a. Since I submitted this report another example of mineralization of the myocardium has been studied from an individual dying of renal insufficiency. In this instance calcium, iron, carbonate and phosphate were all demonstrable.

19. Moog, F.: Biol. Rev. **21**:41, 1946.

20. Lorch, I. J.: J. Bone & Joint Surg. **31B**:94, 1949.

alkalinity has commonly been invoked in the calcification of necrotic tissue (Barr [21]). If this actually is a factor one must conclude that in noncellular regions such as the connective tissue of the choroid plexus, for example, its origin must be a change in the collagen itself.

In the cerebral arteries the initial mineral deposit apparently develops on the elastica (the interna only is present in these arteries), while early the muscle coat seems essentially unaltered. The affinity of iron for the elastica has been demonstrable in vessels under other conditions—for example in the intrasplenic arteries in conjunction with excessive deposits of iron such as occur in hemochromatosis or in congenital hemolytic anemia. Here the elastica, as well as the collagenous fibers in the adventitia in some instances, contains demonstrable iron and phosphate but not calcium or carbonate. Although the major deposit of iron in the spleen under these conditions is of the hemosiderin type, much of which is in phagocytes, that found in the elastica and elsewhere in the wall of the vessels is of the labile variety containing both ferric and ferrous components. It seems plausible that ionized iron was ultimately derived from the destruction of hemoglobin by the phagocytes and that whether it was liberated from the phagocytes or from the interstitial hemosiderin deposits that are found with such large amounts of hemosiderin as occur under these conditions is not known. The presence of inorganic phosphate could account for the basophilia shown by the elastic and collagenous fibers here. Elastic fibers in the skin and the vessels can be demonstrated to become more basophilic with increasing age (unpublished data); this general phenomenon does not appear to be ascribable to the presence of phosphate. Calcium and carbonate have also been present in all the examples of cerebral vessels studied. Hurst,[17] employing indirect methods, concluded that calcium was absent, and in recent years the existence of calcium as well as of iron is still debated by some.[22] Strassmann,[23] who investigated the origin of the iron found in and around cerebral vessel walls, concluded that it frequently arose in the absence of hemorrhage in such regions as the globus pallidus, where these changes were frequent, and that it probably represented an involutional process. When the mineral deposits are small, there appears to be little other change in the structure of the wall of the vessel. With larger deposits there is commensurately greater atrophy of the muscle coat. This appears to be the result rather than the cause of the deposit. In the present material lipids could not be correlated with mineralization of the vessel walls.[24] The cause of the deposits is not in any way elucidated by these studies except for the inference that the deposit probably does not necessarily indicate cell death.

In the choroid plexus the interstitial granules contain iron as well as calcium. The appearance of all of the granules here and in the white matter of the brain is not always the same. Some of them are glassy while others are almost opaque. The former are clearly laminated, and the iron, if present, is confined to the outermost

21. Barr, D. P.: Physiol. Rev. **12**:593, 1932.

22. Biggart, J. H.: Pathology of the Nervous System, Baltimore, Williams & Wilkins Company, 1949. Freeman, W.: Neuropathology: The Anatomical Foundation of Nervous Disease, Philadelphia, W. B. Saunders Company, 1933.

23. Strassmann, G.: J. Neuropath. & Exper. Neurol. **4**:393, 1945; **8**:428, 1949.

24. Klotz, O.: J. Exper. Med. **8**:382, 1906.

layer. In the opaque ones iron is abundant and is usually present throughout the individual granules. The granular deposits elsewhere, for example the Schaumann bodies of Boeck's sarcoid, have properties similar to the latter.

When the iron is confined to the peripheral rim of the granule, this is reminiscent of its localization in a comparable region of recent calcification in the subperiosteum of embryonic bone. MacCallum [4] raised the question whether the iron here was facilitating the calcium deposition as was demonstrable in the precipitation of calcium phosphate in vitro.

In the damaged myocardial fibers in some examples only iron and phosphate were demonstrable. These cells were without nuclei, and it is suggested that the changes might have resulted from the splitting of unused glucose phosphate and its subsequent combination with iron from myoglobin. Another possible source of phosphate could be desoxyribonucleic acid presumably from the nuclei of the muscle cell, which is demonstrable at times, combined within the necrotic myocardial fibers.[25] The examples of human myocardium studied did not contain calcium and might well represent the early stage with the deposit of calcium to follow later as was seen in the experimental material after direct myocardial injury. Suffice it to say that adequate tests for the presence of calcium should be conducted on all such material, rather than assume the presence of calcium from the depth of hematoxylin staining or from a positive von Kossa reaction which gives indication only of such acidic groups as carbonate or phosphate that form insoluble salts with silver.

SUMMARY

Various sites of mineralization in the body have been investigated by direct tests for the presence of calcium, iron, carbonate, and phosphate. Blood vessel walls in the brain and elsewhere and granular deposits in the brain, the choroid plexus, the kidney, and in other organs have all been found to contain calcium, iron, carbonate, and phosphate. Some foci of mineralized myocardial fibers have contained phosphate and iron, while others have yielded positive results in tests for calcium and carbonate as well. The validity of the tests employed is discussed, as well as that of others commonly used.

Technical assistance was rendered by Miss Patricia Reynolds.

25. Bunting, H.: Yale J. Biol. Med. **22**:521, 1950.

Case Reports

SINGLE CORONARY ARTERY

Report of Two Cases

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A SINGLE coronary artery supplying the heart represents an unusual anomaly. At least two possibilities present themselves in the pattern of distribution of such vessels. The first and commoner type of single coronary artery is that in which the vessel originates from a single ostium and immediately branches to give rise to vessels which soon follow the normal course of the right and left coronary arteries. The second type is that described by Hyrtl [1] in 1882 as fulfilling the criteria for a true single coronary artery. In this type the vessel follows the course of a single coronary artery to supply the entire heart. The finding of a case of each of these two variations recently in the course of routine necropsies forms the basis of this report.

The first specimen was from a man, 77, who had died of carcinoma of the esophagus. The heart weighed 325 gm. (Fig., *a*). The ostium of the right coronary artery was absent, and the wall of the corresponding aortic sinus was smooth. The left coronary ostium was normally situated but was of greater diameter than normal, measuring 12 mm. in this dimension. The left coronary artery progressed to the left atrioventricular groove and thence circumferentially to the right atrioventricular groove, terminating near the base of the pulmonary conus. Vessels arising from this single artery coursed in the anterior and posterior interventricular sulci and over the obtuse borders of the heart in the normal pattern of distribution of the anterior and posterior descending and marginal branches of the coronary arterial system. Multiple cross sections of the vessels revealed an area of occlusion in a branch of the anterior descending coronary artery with an underlying healed anteroseptal infarct measuring 4 by 3 by 1 cm. Microscopic examination of sections through the occluded vessel revealed a recanalized thrombus with extensive deposition of iron. The myocardium, the endocardium and the valves were otherwise normal.

The second specimen was from a woman, 44, who died of a widespread malignant lymphoma. The heart weighed 255 gm. and, with the exception of subendocardial petechial hemorrhages and the anomalous coronary vessels, was anatomically normal (Fig., *b*). The right aortic sinus presented a single ostium, from which a single right coronary trunk arose. This vessel branched at a point 3 mm. from its origin to give rise to a right coronary artery with normal distribution for that vessel and a left coronary artery. The latter passed between the base of the aorta and the pulmonary trunk to give rise to anterior descending and left circumflex branches. The distribution of these main branches was then essentially normal after the primary branching of the single coronary artery.

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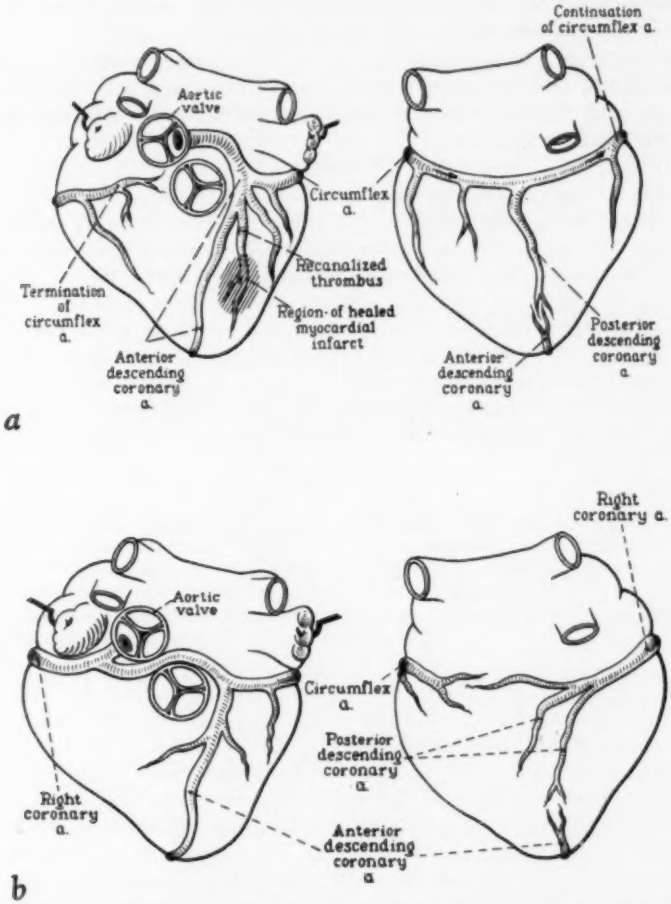
1. Hyrtl, J., quoted by Smith, J. C.: Circulation **1**:1168 (May) 1950.

COMMENT

Smith [2] has recently reviewed the cases of single coronary artery reported in the literature and added two personally observed cases. As Krumbhaar and Ehrich,[3] and others had done, Smith defined a single coronary artery as one in which the entire myocardium is nourished by an artery that arises by one ostium from an arterial



(*a*) Distribution of the single coronary artery in the first case. This type conforms to Hyrtl's postulate for a true single coronary artery. (*b*) Pattern of origin of right and left coronary arteries arising from a single vessel as seen in the second case.

trunk, regardless of the course and pattern of distribution of branches that the vessel may have. Nevertheless, a subclassification in accordance with Hyrtl's criteria for

2. Smith.[1]

3. Krumbhaar, E. B., and Ehrich, W. E.: Am. J. M. Sc. **196**:407 (Sept.) 1938.

a true single coronary artery may be made. The first case presented here is an example of it (Fig., *a*). The second type of single coronary artery gives rise to branches which follow the distribution pattern of a right and a left coronary artery (Fig., *b*). This type, which Hyrtl objected to as possibly being a form of ectopia of the anlage of the "absent" coronary artery, is represented by the second case presented here. The third type which enters Smith's classification is a single coronary artery which has an atypical distribution and which usually is found in association with other congenital cardiac malformations.

The explanation of these anomalies on the basis of either absence or ectopia of the anlage of a coronary artery is at least a good theoretical basis for discussion, although valid conclusions probably cannot be drawn from morphologic study of fully developed hearts alone.

SUMMARY

Single coronary arteries in adults fall into two main groups. In one group the vessel follows the distribution of a single artery to supply the entire myocardium. The other main group displays a single vessel which branches to give rise to arteries which follow the distribution pattern of right and left coronary arteries.

A case of each type is presented.

PULMONARY ADENOMATOSIS

Report of a Case Demonstrating Peculiar Histological Changes in Certain Bronchioles

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IN RECENT years there have been reported several cases of a peculiar disease of the lungs, which has been given different names; mostly it has been called pulmonary adenomatosis or alveolar-cell carcinoma. It is a condition which presents characteristics that distinguish it completely from the usual forms of pulmonary cancer. It has provoked great interest, first, because the lesions resemble those present in the sheep disease *Jagziekte* and, second, because its histogenesis has appeared to be other than one of bronchial origin.

Recently I have been able to study a typical case of pulmonary adenomatosis. Histological sections revealed in the walls of bronchioles structural abnormalities which hitherto I have never seen described. These abnormalities appeared to merit description because of the possible bearing they may have on the histogenesis of pulmonary adenomatosis. A general description of pulmonary adenomatosis has been given so frequently in recent years, and so completely in the publication of Neubuerger and Geever [1] and that of Swan,[2] in particular, that I purpose to omit it from this paper.

CLINICAL HISTORY

A woman, 72, was admitted to the Brugmann Hospital, complaining of dyspnea and a dry cough of two months' duration. Three weeks before her admission she had a paraplegic stroke. She showed profound cachexia, with flaccid paraplegia and complete anesthesia to the costal margins. Radiological examination of the chest showed nodular infiltrations in both lungs. A clinical diagnosis of pulmonary and vertebral metastasis of unknown origin was made. The patient died 10 days after admission.

PATHOLOGICAL OBSERVATIONS

Gross Findings.—Autopsy disclosed numerous adhesions in both pleural cavities. There was a large effusion of clear yellow fluid in the left pleural cavity, with a smaller collection of similar fluid on the right side. Both lungs were small. They contained numerous cherry-sized nodules (Fig. 1), some visible through the pleura. The nodules were white, firm in consistency, and easily distinguishable in the parenchyma. The parenchyma itself, otherwise, appeared normal. Dissection of the principal bronchi failed to display the presence of a bronchial tumor or of bronchiectasis. The hilar and supraclavicular lymph nodes were enlarged and infiltrated. Sagittal section of the vertebrae showed multiple metastases in the

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1. Neubuerger, K. T., and Geever, E. A.: Alveolar Cell Tumor of Human Lung, Arch. Path. **33**:551 (April) 1942.

2. Swan, L. L.: Pulmonary Adenomatosis of Man, Arch. Path. **47**:517 (June) 1949.

vertebral bodies. In the thoracic segment the medullary canal was reduced, and the spinal cord appeared to be compressed. The other organs revealed no relevant gross pathologic changes.

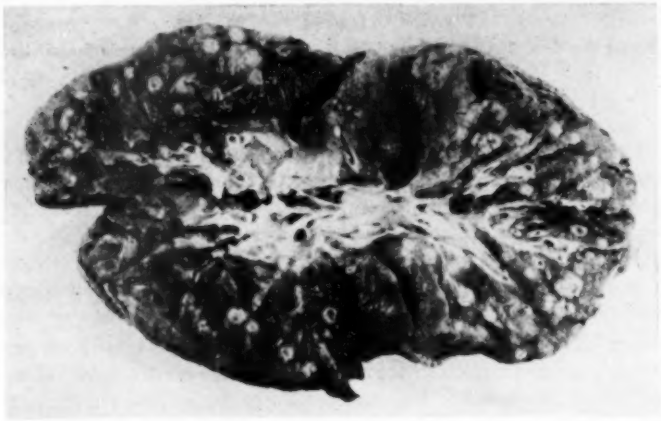

Fig. 1.—Macroscopic aspect of the right lung. Numerous nodules are disseminated in the parenchyma.

Fig. 2.—Section of the tumor showing the alveolar walls lined by a mucus-secreting columnar epithelium with occasional papillary processes.

Microscopic Observations.—Lungs: The nodules showed a proliferation of cells, which lined the alveoli (Fig. 2). The cells formed a single layer, but in some places reduplication was seen in the form of papillary processes, which occupied the lumen

of the alveolus. The cytological aspect was quite uniform; the cells were tall and cylindrical in form, the apical pole sometimes showing a pear-shaped swelling and almost always a clear vacuole. The nucleus was oval and situated near the base or in the center of the cell. The nuclear membrane was quite distinct. A fine chromatin was present. No nuclear pleomorphism or mitotic activity was remarked. Many alveoli were distended with mucus (metachromatic with toluidine blue) containing cellular debris (Fig. 3) and sometimes many round-cell macrophages with foamy cytoplasm and small eccentric nuclei. No transitional changes were remarked between these macrophages and tumor cells. There was no thickening of the interalveolar septa, and there were no signs of inflammatory lesions. The bronchi were normal.

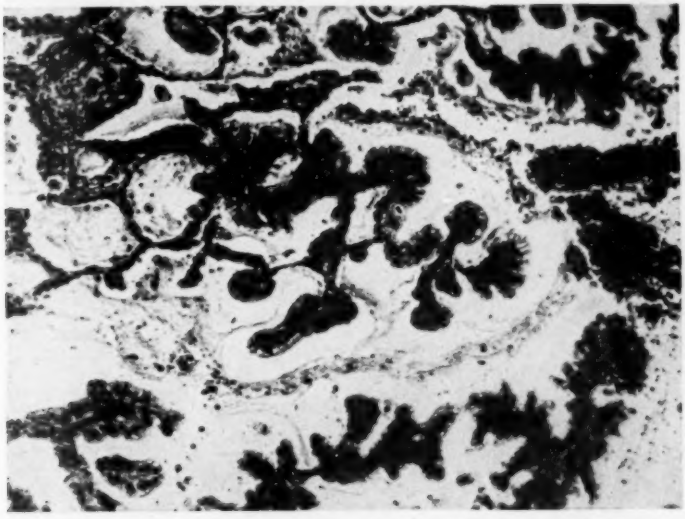

Fig. 3.—Another section of the tumor, showing mucus and cellular debris in the alveolar spaces.

In one nodule examined in serial sections were found peculiar structural abnormalities in the walls of several bronchioles. There were present narrow-necked diverticula (Fig. 4), which extended into the bronchovascular tract, alongside the bronchiole wall in both directions. The lining of these diverticula was in most cases formed by a mucus-secreting cylindrical-cell epithelium (Fig. 4*A*), indistinguishable from that which lined the alveoli in the tumor nodules. In the neighborhood of the diverticular necks this secretory epithelium changed to a columnar or cubic, ciliated epithelium which was continuous with the bronchiolar mucosa (Fig. 4). In several diverticula this ciliated epithelium extended for a considerable distance on the wall of the diverticulum (Fig. 4*B*). In certain bronchioles the normal ciliated epithelium was replaced for a certain distance peripherally and

centrally from the diverticular opening by a columnar, secretory epithelium identical with that of the tumor (Fig. 5*A*). These bronchiolar structural abnormalities were found in only one of the nodules examined.

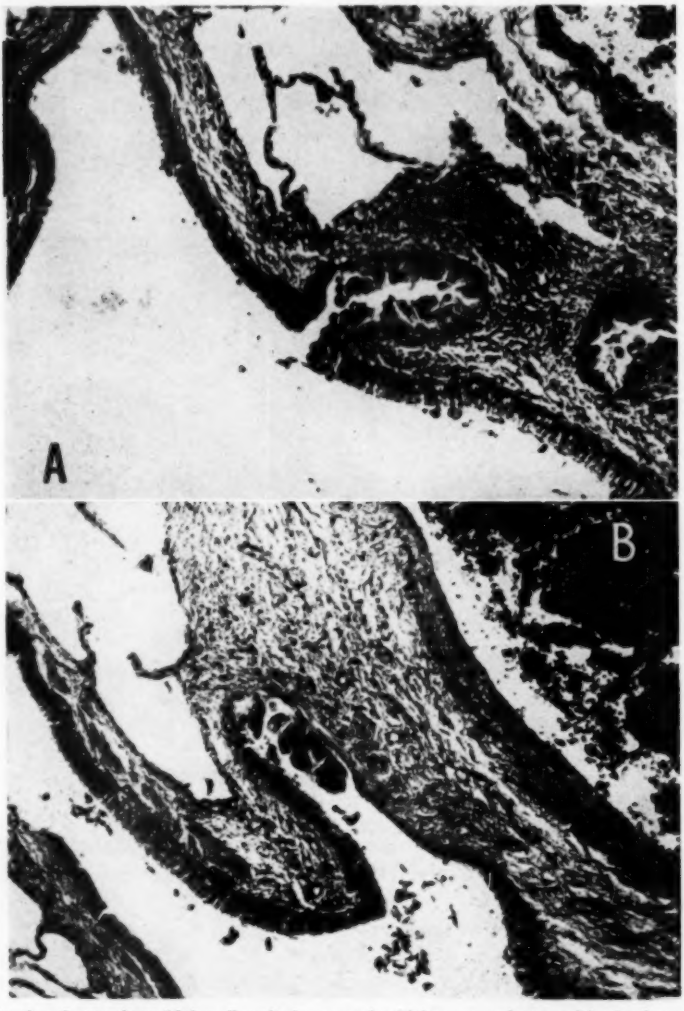

Fig. 4.—*A*, two bronchiolar diverticula, one of which communicates with the bronchiolar lumen. Note the ciliated columnar epithelium on one side of the diverticular neck.

B, another diverticulum taken at its opening into the bronchiolar lumen. Note the ciliated epithelium extending in the wall of the bronchiolar diverticulum.

In the other nodules were many examples of tumor invasion of blood vessels. Here and there in the nodules examined examples of transition between the epithelium of the tumoral bronchioles and a tumor-like epithelium were seen.

Hilar and Subclavicular Lymph Nodes: The different nodes were almost completely invaded by an adenocarcinoma of alveolar structure, the alveoli being regular and lined by a cylindrical epithelium, in which the secretory character was less developed than in the primary tumor. Mitotic figures were rare.

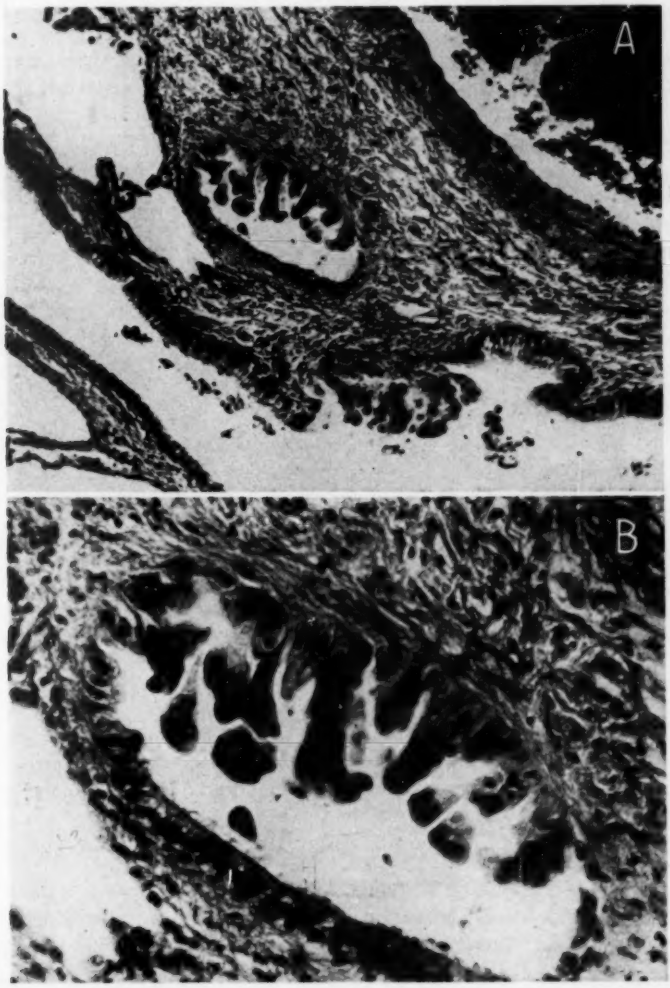

Fig. 5.—*A*, same diverticulum as the one shown in Figure 4*B*, but a few serial sections farther on. The ciliated epithelium of the bronchiole is replaced for a certain distance from the diverticular opening by a columnar tumor-type epithelium.

B, high-power view of the diverticulum of *A*, to demonstrate the detail of the epithelial lining of this diverticulum: columnar tumor-type epithelium in the upper half, ciliated bronchiolar type in the lower half.

Vertebrae: The medullary canals were enlarged and contained a fibrotic marrow. The neoplastic tissue present was arranged in alveoli, the cells of which showed little secretory differentiation (Fig. 6). The trabeculae were narrowed and but slightly calcified.

Other Organs: Microscopic examination of other organs showed no relevant histologic changes.

Pathological Diagnosis.—Pulmonary adenomatosis (nodular type); metastasis involving the hilar and supraclavicular lymph nodes and the vertebrae.

Fig. 6.—Section of a vertebral metastasis.

COMMENT

The case described was a typical example of pulmonary adenomatosis, or alveolar-cell carcinoma. Present were the three criteria of Swan [2] necessary for making a diagnosis of pulmonary adenomatosis:

1. Proliferation of mucus-secreting high-columnar cells lining the alveoli
2. Absence of a primary tumor in the principal bronchi
3. Absence of a possible primary cancer focus in the rest of the body

A complete general description of pulmonary adenomatosis with its different clinical and anatomical characteristics and a complete review of relevant literature were given by Neubuerger and Geever in 1942 and Swan in 1949. Their descriptions are so complete that a repetition appears to be superfluous. However, the present case presents curious pathological features which I think deserve detailed discussion.

Concerning the histogenesis of pulmonary adenomatosis, authors are divided into two groups. The majority admit that the tumors develop from the epithelium lining the alveoli. Others believe that the tumors originate from the epithelium of the bronchioles. In the present case histological studies revealed hitherto unpublished structural abnormalities in the walls of certain bronchioles. There appeared to be present bronchiolar diverticula which could not be mistaken for bronchiolar glands or for tumor-containing lymphatics. The diverticula occurred in the bronchioles which normally are without glands, and microscopically they did not present the structure of a normal bronchial lymph node. Some of the diverticula were lined with ciliated epithelium and so could not be interpreted as a tumor-invaded lymphatic. They could not be confused with an alveolar cavity, because of their situation in the bronchovascular tract, alongside the blood vessels, and the occasional presence of a ciliated epithelial lining. We believe that such diverticula can only be embryological malformations in certain peripheral segments of the bronchial tree.

The similarity between the mucus-secreting columnar epithelium present in some of these diverticula and those cells which constitute the tumor points to a possible site of origin of the neoplastic process.

This is in keeping with other examples of cancerous degeneration in embryonic malformations. I cannot believe that the nodule which was removed was the unique site of the embryological abnormalities, and prefer to think that this abnormal state existed throughout the corresponding part of the bronchial tree. This conception would give support to the more generally accepted multicentric origin of pulmonary adenomatosis.

I have also had the opportunity of examining preparations of the sheep disease *Jagziekte*, but I was unable to see any example of the embryonic abnormality described above. I have remarked in these preparations the thickening of the interalveolar septa, which, according to Cowdry,[3] are an essential occurrence in the disease, but also would like to note that I have not seen the specific septal thickening seen in the present case of human pulmonary adenomatosis.

SUMMARY

Embryological abnormalities were noted in a case of pulmonary adenomatosis, in the form of bronchiolar diverticula. It seemed evident that these were not glandular processes or lymphatics invaded by tumor cells.

These diverticula seemed to be the probable site of origin of the tumor.

Such embryological abnormalities, presumably present throughout the bronchial tree, would give support to the theory of multicentric origin.

A comparison has been made between this and the histological picture of *Jagziekte*.

Professor P. Gerard placed material from a South African case of jagziekte at my disposal.

3. Cowdry, E. V.: Etiology of Jagziekte: Primary Lesions, J. Exper. Med. **42**:323, 1925.

ANOMALOUS CARDIAC BLOOD VESSEL COMMUNICATING WITH THE RIGHT VENTRICLE

Observations in a Case of Pulmonary Atresia with an Intact Ventricular Septum

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AND
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CONGENITAL malformations of the coronary blood vessels include aneurysms, arteriovenous fistulas, and abnormal origins of the arteries which have been found arising from the aorta or from the pulmonary trunk or a pulmonary artery. A coronary blood vessel arising from a cardiac ventricle, as far as we know, has not been previously recorded.

This report describes an instance of a blood vessel emerging from the right ventricle in a heart with pulmonary valvular atresia and intact ventricular septum.

DESCRIPTION OF SPECIMEN

The heart was obtained from a female infant, aged 4 days, who for two days had suffered from episodes of cyanosis during crying and feeding. Roentgenographic examination of the thorax had shown evidence of cardiac enlargement. Murmurs or thrills had not been present. The parental history, the gestation, and the delivery had not been abnormal.

At necropsy there was symmetrical globular enlargement of the heart. A large coronary blood vessel with a pale yellow wall was seen to emerge from the myocardium of the anterior right ventricular wall halfway between the apex and the base of the heart (Fig. 1*a*). It extended inferiorly, parallel with the anterior longitudinal sulcus, over the apex of the right ventricle to anastomose with the posterior descending coronary artery, and terminated by anastomosis with descending branches of the left circumflex coronary artery (Fig. 1*b*). Its greatest diameter measured 0.3 cm. The right coronary artery originated normally from the right aortic sinus, extended forward between the right auricle and the pulmonary trunk to the right atrioventricular sulcus, and coursed circumferentially to terminate as the posterior descending coronary artery within the posterior longitudinal sulcus. The left coronary artery arose normally from the left aortic sinus. It extended between the left auricle and the pulmonary trunk to the upper end of the anterior longitudinal sulcus, where it terminated by branching into the anterior descending coronary artery and the left circumflex coronary artery. The distribution of these vessels was normal.

Pulmonary valvular atresia was present. The pulmonary cusps were completely fused to form a diaphragm-like membrane. Vestigial cuspal margins were represented by three raphe-like thickenings situated symmetrically within the superior aspect of the membrane and extending from its center radially to the pulmonic ring. The pulmonary trunk measured 0.7 cm. in diameter except at the level of the valvular ring, where it measured 0.5 cm. in diameter. The chamber of the right ventricle was small, being 2.8 cm. deep, and its volume was estimated to be

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one-sixth that of the left ventricle. Compared with the diminutive size of the right ventricular chamber, the right ventricular wall was disproportionately thick, measuring 0.8 cm. The ventricular septum was completely formed. The tricuspid orifice was narrow, measuring 0.5 cm. in diameter. The leaflets, though uniformly small, were otherwise unaltered. The right atrium and its appendage were conspicuously dilated. The connections with the superior vena cava, the inferior vena cava, and the coronary sinus were normal. The foramen ovale was patent. Its valve was normally placed but was thickened on the free margin and rolled to the left.

The left atrium was moderately dilated. The cavity of the left ventricle measured 3.2 cm. in depth. Its wall was 0.6 cm. thick. No abnormalities could be found in the mitral and aortic valves. Uniform enlargement of the aorta was present; its supravalvular external diameter measured 1.2 cm. The ductus arteriosus was patent, but its lumen measured only 0.2 cm. in diameter.

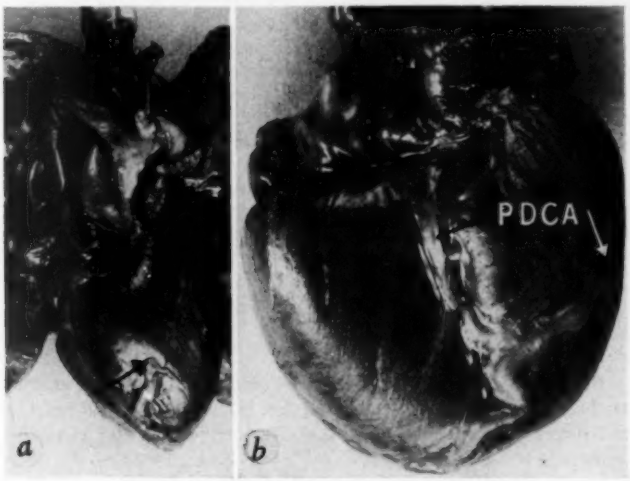

Fig. 1.—(*a*) right anterior view of the heart. An anomalous coronary vessel emerges from the wall of the right ventricle (arrow). (*b*) Right posterior view of the heart. The anomalous coronary vessel emerges from the wall of the right ventricle (white arrow). This vessel courses inferiorly to anastomose with the posterior descending coronary artery (*PDCA*) and then progresses downward over the apex, where it anastomosed with a branch of the left circumflex coronary artery.

In order to establish continuity of the chamber of the right ventricle with the lumen of the anomalous cardiac vessel, injections of water and aqueous suspensions of dye were performed by placing the tip of a hypodermic syringe through the tricuspid orifice of the unopened heart. When the right ventricular chamber was filled with fluid, the anomalous vessel ballooned under slight pressure, as did also the aforementioned anastomotic branches of the coronary arteries. When dye substances (methylene blue and India ink) were injected into the chamber of the right ventricle, the following structures became stained: the lining of the anomalous coronary vessel, the intima of all major branches of both coronary arteries, the endocardium of the right ventricle, the intima of the aorta immediately around the coronary ostia, and the myocardium of both ventricles. Despite the fact that the anterior descending branch of the left coronary artery was stained, one could not definitely establish whether this was due to its having an actual direct anastomosis with the anomalous coronary vessel, or to reflex of the dye substances by way of the left circumflex coronary artery. The linings of the right and left atria, the left ventricle, and the pulmonary trunk were not stained.

Stained histologic sections were prepared serially in an area extending from 5 mm. below to 5 mm. above the level at which the anomalous coronary vessel emerged from the right ventricular wall. Other sections were taken at intervals of 5 mm. through this vessel toward the apex. Further sections were made to include both coronary arteries, their branches, and the left ventricular wall.

Endothelium-lined sinusoids were numerous within the myocardium of the right ventricle; they readily communicated with the lumen of that ventricle (Fig. 2*a* and *b*). Their walls were composed of thin, laminated collagen fibrils; in the outer half of the sinusoidal wall there were interspersed elastic fibrils as well. In the thicker walls of the larger sinusoids the elastic tissue was formed into a distinct lamina at the outer margins. By serial sections, sinusoidal connections could be traced directly from the lumen of the right ventricle through the myocardium to the lumen of the anomalous cardiac vessel (Figs. 2*a* and *b* and 3*a*). As the area of transition from a large sinusoid into the anomalous coronary vessel was reached, gradual thickening of the fibroelastic layer occurred and was accompanied by gradual addition of an outer collagenous layer. The elastic lamina of the sinusoid became gradually fused with the inner fibroelastic layer of the anomalous cardiac blood vessel. This vessel was composed of an inner fibroelastic layer lined by endothelium and an outer collagenous layer (Figs. 3*b* and 4*a*).

Muscle fibers were not identified within the anomalous coronary vessel at any level. An anastomosis could be demonstrated between this vessel and a branch of a coronary artery in serial sections (Fig. 4*b*). The inner fibroelastic layer of the anomalous vessel gradually thinned to become continuous with the arterial intima. Similarly, the outer collagenous layer became continuous with the adventitia. The muscular media of the artery by degrees appeared between the two layers at the area of transition between the two vessels.

No histopathologic changes were seen in sections of the right and left coronary arteries or of the left ventricle other than the presence of India ink in the myocardial capillaries and against the lining of the arteries (Fig. 4*c* and *d*).

COMMENT

In 1898 Pratt[1] demonstrated Thebesian vessels emerging from persistent ventricular communications. Lewis[2] in 1904 recognized sinusoids in the walls of embryonic hearts. Later Blakeway[3] and Reid[4] each described hearts in which they found direct communications between the left ventricular chamber and the left coronary artery.

A careful description of intertrabecular sinusoids of the myocardium of the rabbit embryo was recorded by Grant[5] in 1926. He showed that the lumens of these sinusoids are continuous with the neighboring cardiac chambers and that the sinusoidal endothelium is continuous with the endocardial endothelium. Ultimate connections with the coronary arteries and veins develop by progressive outgrowth of the sinusoids. He further demonstrated that in the embryo the myocardial sinusoids are reduced to capillaries as the myocardium condenses during growth. The Thebesian system and the epicardial capillaries represent specialized derivatives of the embryonal sinusoids.

Simultaneously Grant[6] reported a case of pulmonary atresia associated with a small aneurysm of the right ventricular wall. The aneurysm communicated by way of sinusoids with the chamber of the right ventricle on one hand and with

1. Pratt, F. H.: Am. J. Physiol. **1**:86, 1898.
2. Lewis, F. T.: Anat. Anz. **25**:261, 1904.
3. Blakeway, H.: J. Anat. **52**:354, 1918.
4. Reid, Charles: J. Anat. **57**:12, 1922.
5. Grant, R. T.: Heart **13**:261, 1926.
6. Grant, R. T.: Heart **13**:273, 1926.

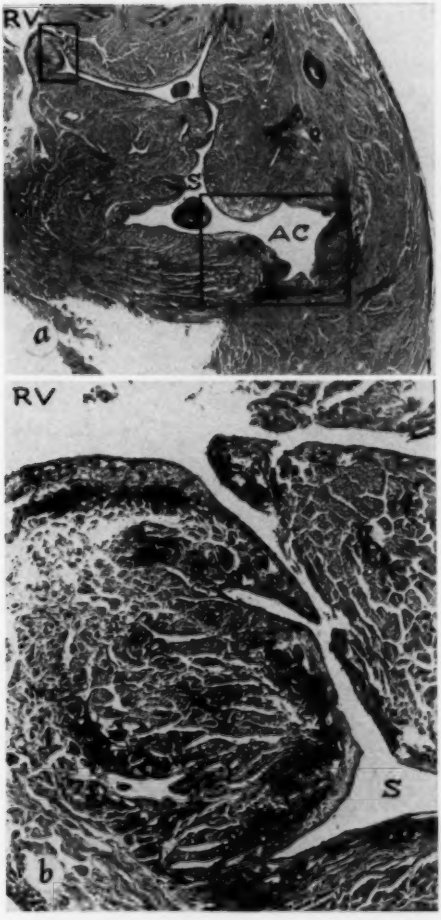

Fig. 2.—Serial sections of the right ventricle. Verhoeff's elastic tissue stain counterstained with Van Gieson's connective tissue stain. (*a*) Serial section No. 159 showing a sinusoid of the right ventricular myocardium. At this level (within smaller rectangle) the sinusoid is separated from the right ventricular chamber (*RV*) only by a slender band of connective tissue lined on either side by endothelium. The large open space (within larger rectangle) is the orifice of the anomalous coronary vessel (see Fig. 3*a*) (x 7). (*b*) Serial section No. 181. This section is slightly nearer the base of the heart than that illustrated in *a*. The general region illustrated is shown within the smaller rectangle in *a*. Continuity of the right ventricular chamber (*RV*) with a sinusoid (*S*) is demonstrated (x 70).

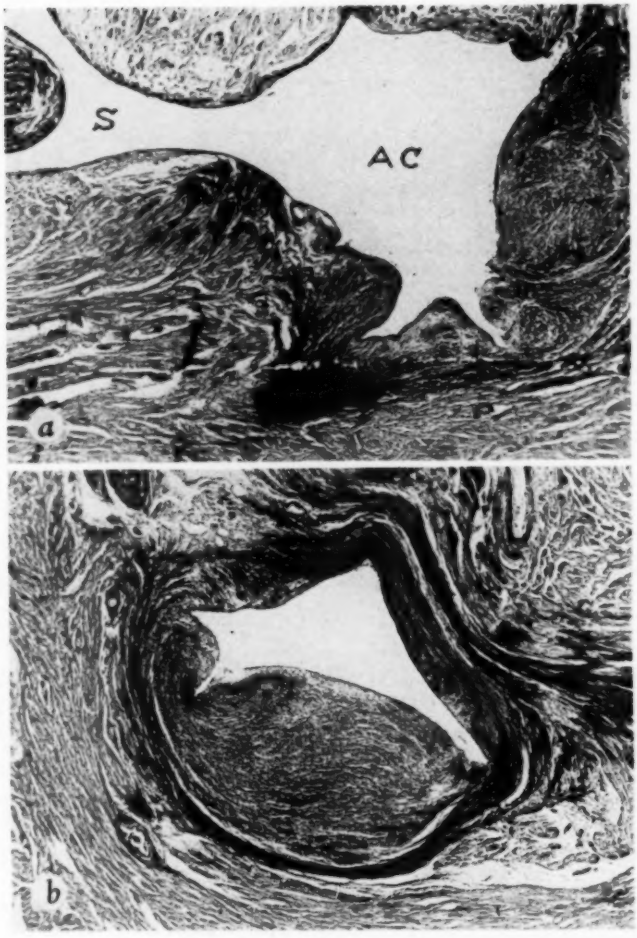

Fig. 3.—Sections of the right ventricle. Verhoeff's elastic tissue stain counterstained with Van Gieson's connective tissue stain. (*a*) Serial section No. 159. Higher power magnification of the tissue shown within the larger rectangle in Figure 2*a*. A sinusoid (*S*) of the right ventricular myocardium is shown emptying into the orifice of the anomalous coronary vessel (*AC*). The ovoid mass in the wall of the anomalous vessel is composed of fibroelastic tissue (x 28). (*b*) Anomalous coronary vessel in the right ventricular myocardium at a level 1.6 mm. nearer the apex of the heart than the level illustrated in *a*. At this level there is a distinct lumen although it is eccentrically placed because of the localized density of the fibroelastic tissue (x 30).

the coronary arteries, veins, and capillaries on the other. He interpreted the blood-filled spaces as being persistent embryonal sinusoids, their persistence being due to an elevation of the right ventricular pressure.

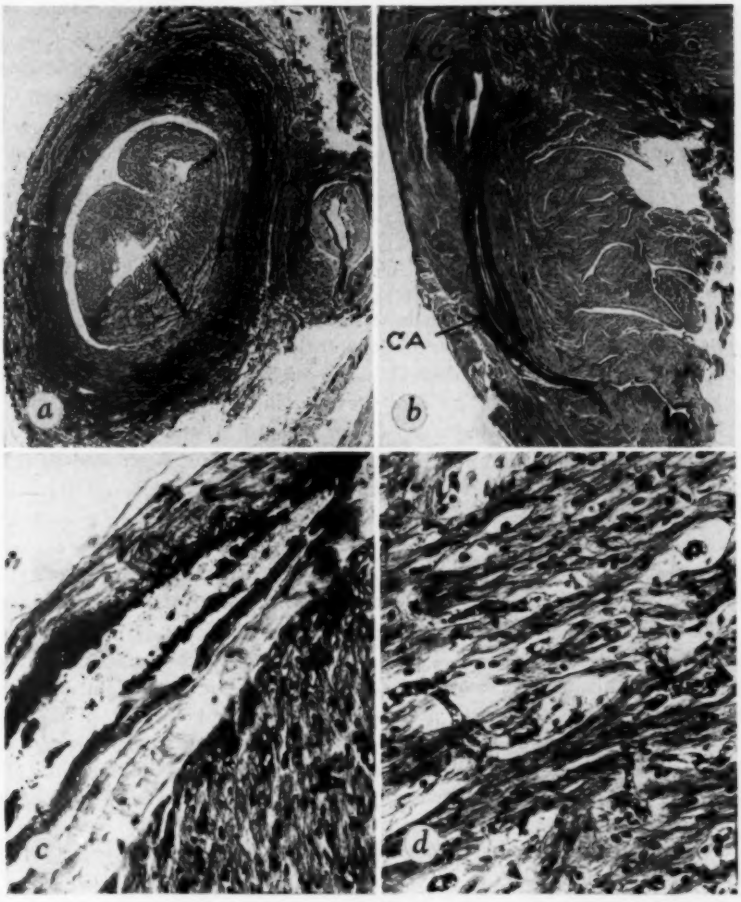

Fig. 4.—Sections of the right and left ventricles (*a* and *b*). Verhoeff's elastic tissue stain counterstained with Van Gieson's connective tissue stain; (*c* and *d*), hematoxylin and eosin stain. (*a*) Anomalous coronary vessel. This section is of a level 10 mm. nearer the apex of the heart than that shown in Figure 3*b*. The anomalous coronary vessel has emerged from the myocardium and lies in the epicardium. Inner fibroelastic and outer collagenous layers are present. (Separation and indentation of the inner layer are artefacts.) (x 26). (*b*) Serial section No. 13. Anomalous coronary vessel in the right ventricular myocardium. A branch of a coronary artery (*CA*) anastomoses with the anomalous vessel (*AC*) (x 7). (*c*) Branch of the left circumflex coronary artery. The lumen contains particulate dye matter which had been injected into the right ventricular chamber (x 215). (*d*) Posterior wall of the left ventricle. Dye particles can be seen partially filling several of the capillaries (x 215).

In 1932 Bellet and Gouley [7] reported a case of aortic atresia with intact ventricular septum in which wide sinusoids were found within the myocardium of the left atrium and ventricle. These sinusoids, interpreted by Bellet and Gouley as persistent embryonal sinusoids, communicated with the chambers of the left auricular appendage and left ventricle on one hand and with the lumen of the left coronary artery on the other. Elevated blood pressure within the left side of the heart and compensation for diminished coronary circulation as a result of the aortic atresia were suggested as causing persistence of the embryonal sinusoidal structures.

Abnormal vascular patterns in the latter two cases are developmentally similar to those in the case that we are reporting. One exception is that a gross blood vessel of sinusoidal origin, present in our case, was not present in the case of Grant or in that of Bellet and Gouley. The aneurysm in Grant's case, however, is probably homologous with the anomalous coronary vessel of our case.

Later Proescher and Baumann [8] and Lyon and associates [9] each recorded a case in which the left coronary artery arose from the pulmonary artery and in which persistent embryonal sinusoids were found within the myocardium of the left ventricle. No anastomotic connections of the sinusoids were described. The former authors postulated that these sinusoids might serve as compensatory circulatory channels in the case of reduced pressures in the left coronary artery.

In 1939 Burchell,[10] studying a dog's heart, found wide endothelium-lined sinusoids in the left ventricular wall. These appeared to have functioned as direct communications between arteries and veins.

The anomalous cardiac vessel described herein is believed to be a specialized structure of persistent embryonal sinusoidal origin. Its histologic composition is neither that of an artery nor that of a vein. Rather, it resembles the embryonal sinusoids which form its inner communication with the right ventricular lumen.

Pulmonary atresia, intact ventricular septum, and a normally functioning tricuspid valve are factors associated with right ventricular hypertension and hypertrophy. It is postulated that because of elevated pressure within the right ventricular chamber the sinusoids were in persistent use carrying blood out of the right ventricle and did not involute. Further, it is believed that the anomalous coronary blood vessel developed as a result of this particular function of the sinusoids. The anomalous coronary blood vessel possibly served during systole to lessen the force of the right ventricular contraction on the blood contained in that ventricle. In so doing it acted as a by-pass through its anastomoses with the coronary arteries and myocardial capillaries.

During early diastole, it is assumed, the pressure in the anomalous vessel was higher than the pressure in the right ventricle. Under this circumstance, at this period, the flow could have been directed from the anomalous vessel into the chamber of the right ventricle.

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SUMMARY

This report is based on the finding of an anomalous vessel arising from the right ventricle in a case of pulmonary atresia with intact ventricular septum in the heart of a female infant, aged 4 days. The anomalous vessel communicated on one hand with the right ventricular chamber by means of sinusoids, and on the other hand with the normal coronary arterial and capillary tree.

It is suggested that the anomalous coronary vessel is derived from embryonal sinusoids.

ADDENDUM

Since the time of preparation of this manuscript Dr. W. Stewart Alexander and Dr. Howard C. Green, of Regina, Sask., Canada, have observed a similar case, which they will report. In their patient there was pulmonary valvular atresia associated with a small ventricular septal defect. A gross communication existed between the right ventricular chamber and the lumen of the left coronary artery.

News and Notes

First International Congress of Neuropathology.—On Sept. 8, 1952, the First International Congress of Neuropathology will meet in Rome, Italy. Dr. Mario Gozzano, professor of neurology and psychiatry at the University of Rome, was elected president of the congress. Dr. Armando Ferraro, of New York, was elected secretary-general. The meeting will last six days. In the morning sessions, assigned topics will be presented by qualified investigators from various countries. In the afternoon sessions, discussions and individual papers dealing with the morning topics will be presented. The following topics have been selected: first day, pathology of demyelinating diseases; second day, pathology of vascular diseases; third day, pathology of schizophrenia; fourth day, pathology of mental deficiencies; fifth day, pathology of senility, and sixth day, special histopathological and histochemical techniques. Neuropathologists, neurologists, psychiatrists, and pathologists are cordially invited. The registration fee for active membership in the congress has been established at $15. Additional information may be obtained from: Dr. Joseph H. Globus, chairman, United States National Committee, 960 Park Ave., New York.

Retirement.—Dr. Frederic Parker Jr. retired on July 18, 1951, from the position of pathologist-in-chief of the Boston City Hospital, a position he has held since the retirement, in 1932, of the late Frank Burr Mallory. Dr. Parker also has served as associate professor of pathology at the Harvard Medical School. He will continue his investigative work at the Boston City Hospital, where he now holds the position of consulting pathologist. Dr. G. Kenneth Mallory, professor of pathology and chairman of the department, Boston University School of Medicine, and lecturer in pathology, Harvard Medical School, succeeds Dr. Parker as pathologist-in-chief at the Boston City Hospital and director of the Mallory Institute of Pathology.

Wisconsin Society of Pathologists.—The Wisconsin Society of Pathologists held their annual meeting on Oct. 3, 1951. Dr. Walter H. Jaeschke, Madison, was elected president; Dr. Edward A. Birge, Milwaukee, vice-president, and Dr. Robert S. Haukohl, Milwaukee, secretary-treasurer. Dr. S. E. Gould of Eloise, Mich., served as moderator at a seminar on "The Pathology of Myocardial Infarction."

Appointment.—Dr. William Boyd, professor of pathology at the University of Toronto since 1937, has been appointed the first professor of pathology in the new Faculty of Medicine of the University of British Columbia, Vancouver, B. C., Canada. He began this appointment Sept. 1, 1951. He has been succeeded at the University of Toronto by Dr. John D. Hamilton, formerly professor of pathology at Queen's University, Kingston, Ont. Dr. Robert H. More, formerly Miranda Fraser professor of comparative pathology at McGill University, has been appointed to fill the vacant chair of pathology at Queen's University.

Reorganization.—The International Society for Geographic Pathology, whose activities were interrupted by the war, has been reorganized. Infectious hepatitis will be the subject for the next conference to be held in Liége, Belgium, July 15 to 18, 1952. The national committees in various countries are now collecting data on this subject, analyses and discussions of which will be presented at the conference. Additional information may be secured from Virgil H. Moon, M.D., secretary, Medical Research Unit, University of Miami, Coral Gables, Fla.

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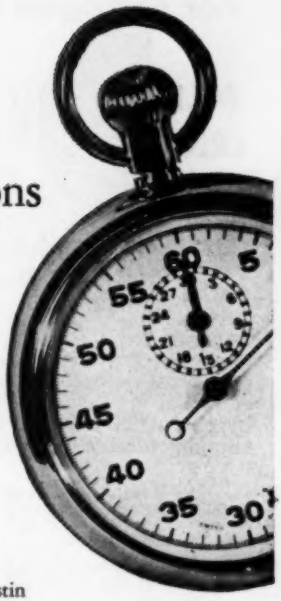

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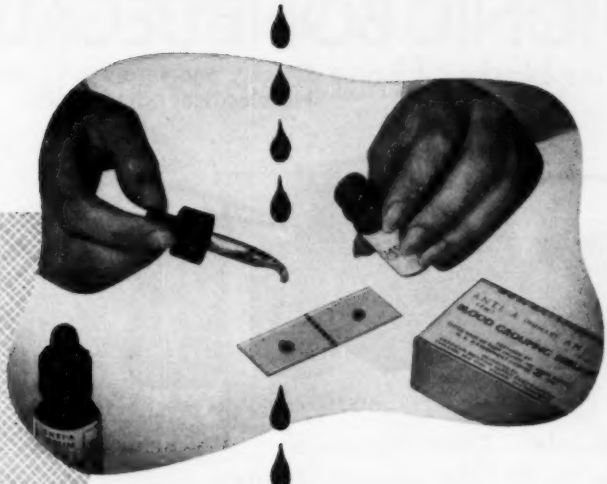

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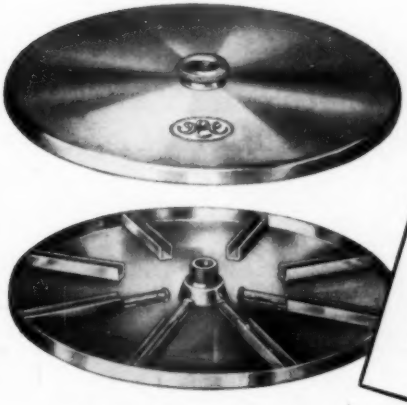

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